THE PHOTOFLUOROMETRIC ESTIMATION OF ESTROGENS AND ANDROGENS

Gordon Arnold Groves, B.Sc.

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TABLE OF CONTENTS

I. Introduction 1 - 16

A. Historical Background 1

B. Chemistry and Nomenclature of the Estrogens 2

C. Extraction of Estrogens From Urine 7

D. Assay of Estrogenic Hormones 9

1. Biological Assay Procedures 9

2. Chemical Assay Methods 12

II. The Problem 17

III. Procedure 18 - 24

A. The Apparatus 19

B. Selection of the Filters 21

C. Standard Reference Solution 22

D. The "Blank" 23

E. Reagents and Equipment 24

IV. Experimental

A. Section One 25 - 110

1. Effect of Acid on Fluorescent Intensity 25

2. Effect of Solvent on Fluorescent Development 28

3. Effect of Temperature on Readings 32

4. Determination of Reaction Time 33

5. Determination of Reaction Temperature 34

6. Technique One 34

7. Results Obtained with Technique One 37

8. Graph One 40

9. Progesterone 41

10. Chart of Progesterone and the Androgens 42

TABLE OF CONTENTS

11. Fluorescence of Testosterone 43

12. Variables Affecting the Fluorescence of Androgens 44

13. Technique for the Photofluorometric Estimation of Androgens 45

14. Graph Two 50

B. Section Two 51 - 61

1. Jailer's Procedure 51

2. Further Investigation of Variables Affecting the Fluorescence of Estrogens 54

3. Technique Two 58

4. Results Obtained with Technique Two 59

5. Graph Three 60

6. Comparison of Sulfuric and Phosphoric Acids 61

C. Section Three 62 - 75

1. Discussion 62

2. Collection of Samples 63

3. Initial Extraction Procedure Employed 63

4. Biological Assay of Extracts 65

5. Fluorometric Analysis of Extracts With Technique One 67

6. Fluorometric Analysis of Extracts With Technique Two 68

D. Section Four 76 - 83

1. Bates and Cohen's Procedure 76

2. Technique Three 78

3. Results Obtained with Technique Three 79

4. Graph Four 80

TABLE OF CONTENTS

E. Section Five 83 - 110

1. Discussion of Commercial Preparations of Estrogens 83

2. Assay of Estradiol Tablets 84

3. Assay of Estrone Aqueous Suspensions 86

4. Assay of Solutions of Estrone in Oil 93

5. Assay of Solutions of Estradiol Monobenzoate in Oil 102

6. Assay of Preparations of Conjugated Estrogens 108

IV. Summary 111

V. Bibliography 112

INTRODUCTION

HISTORICAL BACKGROUND

Early investigation of estrogenic substances was made difficult by the lack of a suitable method of assay. With the introduction of the vaginal smear assay procedure (1) a quantitative test for estrous-producing extracts became available. This made possible the rapid developments in sex endocrinology which followed.

Workers were still hampered at this time by the absence of a cheap and easily available source of estrogens. Using the method of assay introduced by Allen and Doisy (1), Ascheim and Zondek (2) in 1927 provided this source by their discovery that the urine of pregnant women contained large amounts of active principle which was readily extractable with ether and other fat solvents. The fundamental importance of this discovery is strikingly shown by the fact that within three years extensive research culminated in the isolation of a crystalline estrogenic substance almost simultaneously by four different laboratories (3-6). Doisy, Veler and Thayer (3) found the qualitative physiological effects of their crystals to be indistinguishable from those of potent extracts of ovarian follicular fluid. Subsquently, other crystalline compounds exhibiting similiar characteristics have been isolated (7, 8).

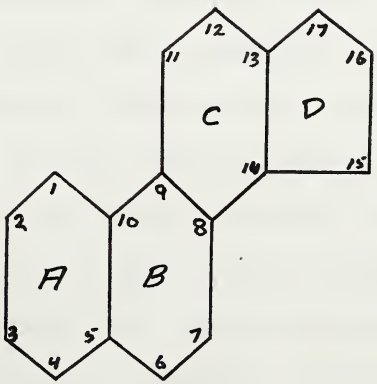
These discoveries gave further impetus to the development of other assay procedures and to investigation of the significance of the various levels and forms of excretion of the estrogens in normal, pathological and pregnacy urines.

THE CHEMISTRY AND NOMENCLATURE OF THE ESTROGENIC HORMONES

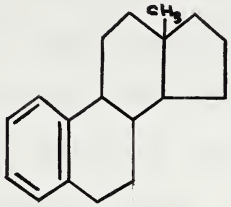
An estrogenic substance is the generic name suggested for any material producing sexual excitement in the female of the lower animals (9). In the human, a number of chemically distinct and biologically active estrogenic substances have been isolated (3-8, 10-12).

A variety of names were suggested for the estrogenic hormone to which Doisy et al (3) had originally affixed the root "Theel-". To avoid confusion the Council on Pharmacy and Chemistry of the American Medical Association (13) in 1936 made official the root "Estr-" which had been proposed earlier (14). A system of nomenclature is outlined below.

In common with other members of the sex hormone family, bile acids, toad poisons and other steroids, the estrogens are derivatives of cyclopentanophenanthrene, a saturated tetracyclic hydrocarbon. The position of each carbon in the formula is numbered, as are the four rings, for convenience in descriptive nomenclature of the various derivatives of this compound.



The parent nucleus of the estrogenic hormones is a perhydro derivative of 13-methyl, cyclopentanophenanthrene in which ring A contains three unsaturated linkages. This nucleus was termed Estrane by Adams et al (14) and chemically it is △ 1, 3, 5- estratriene.



The principle estrogen is alpha-estradiol which is also known as the "true or active" follicular hormone because for many years it was the only active compound isolated in crystalline form from the ovaries. The chemical name is △ 1, 3, 5, - estratriene -3, 17 - trans diol. A stereoisomeric substance is known as beta-estradiol. Another name used to refer to alpha-estradiol is dihydrotheelin.

The two hydroxyl groups constitute the only structural differences between estradiol and the other two well known estrogens, estrone and estriol. The former has only one hydroxyl group in position 3 and a keto group in position 17; the latter an additional hydroxyl group in position 16. Other common names by which these compounds are often designated, as well as their chemical terminology, are given in Chart One.

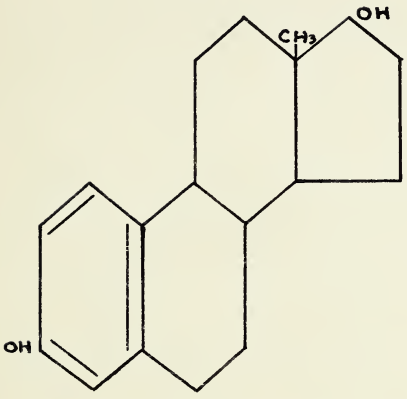
Subsequent to 1930 many estrogenic products derived from mare's urine have been made available commercially. Such products consist either of pure, free estrone or of mixtures of free estrone and the other estrogens normally found in pregnant's mare urine, principally equilin, equilenin, alpha-estradiol and

potassium estrone sulfate (10-12).

Miescher et al (15), taking advantage of the alcoholic nature of the estrogens prepared numerous esters of estrone and estradiol. The sites of esterification are the hydroxyl groups, of which we have two in estradiol at the 3 and the 17 positions and in estrone at the 3 position only. It was found that the dipropionic acid ester and the mono-benzoic acid ester of estradiol greatly enhanced its physiological activities. The structural formulae of these compounds, together with their chemical terminology, are also illustrated in Chart One.

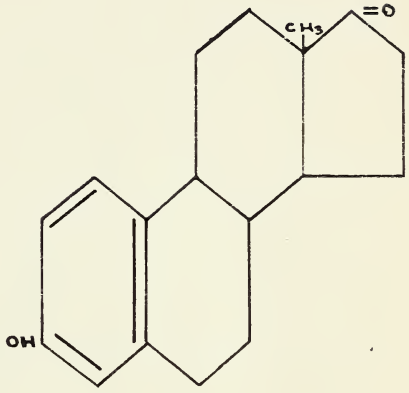
CHART ONE

STRUCTURAL FORMULAS OF THE ESTROGENS



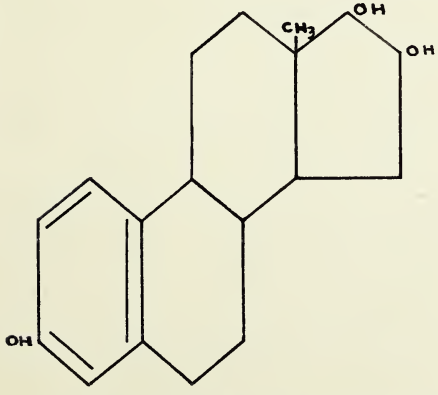
1,3,5-ESTRATRIENE-3,17-DIOL

ESTRADIOL
DIHYDROTHEELIN



1,3,5-ESTRATRIENE-3-HYDROXY-17-KETO

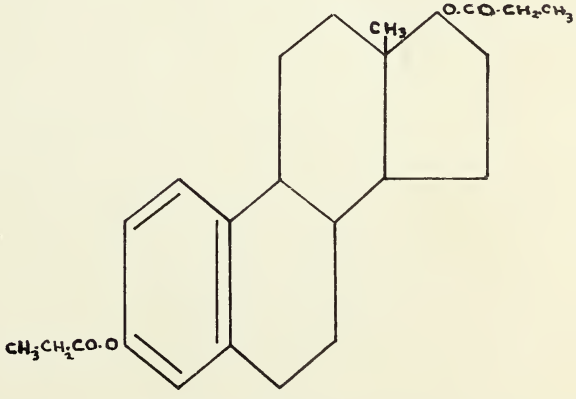
ESTRONE
THEELIN



1,3,5-ESTRATRIENE-3,16,17-TRIHYDROXY

ESTRIOL

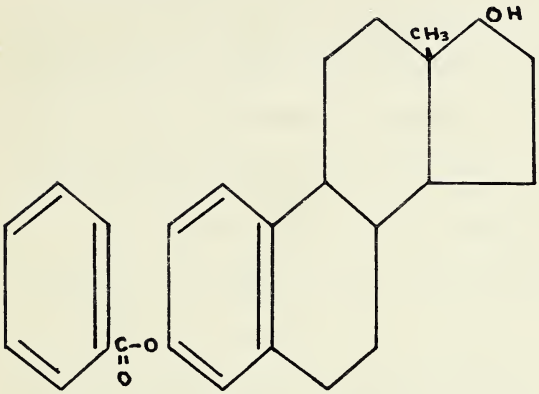
THEELOL



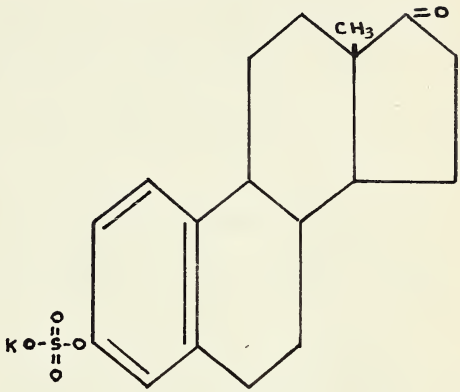
ESTRADIOL
DIPROPIONATE

CHART ONE

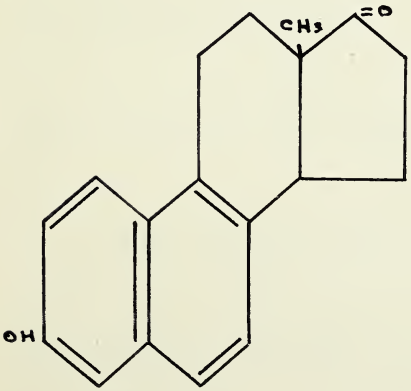
STRUCTURAL FORMULAS OF THE ESTROGENS



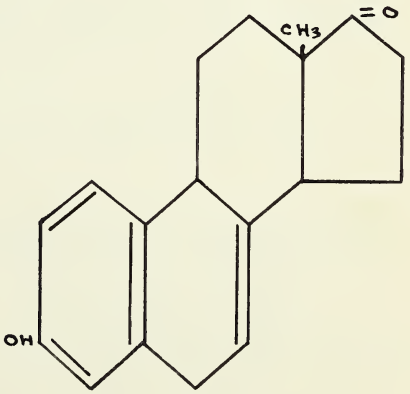
ESTRADIOL MONOBENZOATE



POTASSIUM ESTRONE SULFATE



1,3,5,6,8-ESTRAPENTAENE EQUILENIN



1,3,5,7-ESTRATETRAENE EQUILIN

EXTRACTION OF THE ESTROGENS FROM URINE

In common with other extraction processes involving biological materials, the preparation of estrogenic concentrates consists of three main steps. The first step involves the conversion of the estrogens into a form in which they may be readily extracted. The second of these is the extraction of the hormone, or hormones, together with other lyosoluble substances and the removal of the bulk of the extraneous material from the extract. Thirdly, the resulting concentrate must be purified.

Within recent years Marrian and collaborators (16, 17) have established that over ninety percent of the female sex hormones in human and equine pregnacy urines are excreted, not as the ether-soluble, highly active free hormones, but as water-soluble, ether-insoluble conjugates, relatively inert physiologically. Before a reliable estimation of the total estrogenic content can be obtained by biological assay, it is therefore necessary to subject urine to a form of hydrolytic treatment that will convert all the impotent conjugates into active free estrogens. Similarly, the isolation of the free estrogens in maximum yield for assay first requires the complete conversion of the combined forms into the ether-soluble free forms.

Since Marrian (18) noted that there was a considerable increase in the yields of estrogenic hormones as a result of preliminary treatment with acid, efforts have been concentrated on determining the optimum conditions for hydrolysis. With few exceptions it is generally agreed that hydrochloric acid gives the most satisfactory recoveries (16, 17, 19, 20). Smith et al (20) are of the opinion that boiling the urine for ten minutes

under a reflux condenser with fifteen percent hydrochloric acid satisfies all the criteria for efficient and complete hydrolysis.

The extraction of estrogens from urine has been the subject of investigation by numerous workers (2-8, 10-12, 17, 21-25). The estimation of the estrogenic substance present in urine necessitates its removal from relatively large amounts of fluid. This has, in general, been accomplished by subjecting the urine to a continuous extraction procedure with one of a large number of organic solvents using specially designed apparatus (23, 24). Some workers have attempted to shorten the extraction procedures by extraction of a concentrate, or of an aliquot, of the urine with various organic solvents in a separatory funnel (21, 25). One procedure is also outlined in the literature involving the precipitation of the estrogens from their solution in the urine by the addition of sodium tungstate to form an insoluble complex with the substance being extracted (22).

An essential factor in the extraction of any biological substance is to obtain that substance in a quantitative yield and in a form ready for assay. In the case of estrogens, the possibility of achieving these conditions, in most cases, involves the separation of this estrogenic material from other urinary steroids, particularly androgens. Smith and Smith (26) found that their initial benzene extract of urine, when assayed biologically, was not as active as the extract after further purification by the method of Cohen and Marrian (16). They attributed the lower initial potency to the presence of estrogen inhibitors, persumably androgens, which were removed on purification.

Purification procedures have been developed involving fractionation between immiscible solvents (20, 21), chromatography (27); by using specific chemical reactions of the estrogens (28, 29) to effect their removal from contaminants.

The presence of interfering pigments effects seriously the colorimetric assay of estrogens (21, 25, 27). Various procedures have been devised in attempts to obtain colorless extracts and extracts free from compounds that will give the same colour reaction as the estrogen being investigated. Butyl alcohol (19, 27) has been recommended as an excellent medium for the extraction of estrogens, since urinary pigments are relatively insoluble in this liquid and are not extracted. Bachman and Petit (21) recommend washing the extract with dilute sulfuric acid to remove pigmented and chromogenic materials. Stimmel (27) has evolved a chromatographic method of purification involving the adsorption of estrogens on Alumina (Brockman). The estrogens are subsequently removed with a benzene:methanol mixture, the chromogens and interfering substances being left behind in the adsorption column.

THE ASSAY OF ESTROGENIC HORMONES

Investigation of urinary estrogenic extracts has contributed much to an understanding of the elaboration of these substances and their physiological functions in the organism.

(1) Biological Assay Procedures

Allen and Doisy (1) in 1923, first devised a satisfactory assay procedure for estrogens. This method was the vaginal smear techique and it is still one of the most widely used methods of assay for both natural and synthetic estrogens.

The test is based on the findings that the vaginal epithelium

undergoes a characteristic desquamation during the height of the estrous cycle (30). Atropy of the ovaries or their removal by double ovariectomy will bring about the cessation of this phenomenon. The injection of sufficient estrogenic substance into the spayed female will cause the reappearance of the estrous cycle and once again the vaginal epithelium will become thickened and cornified. These cornified vaginal layers which appear desquamate in much the same manner as do the superficial cornified layers of the skin. Microscopical examination of a sample of vaginal fluid, removed by means of a loop of wire or a pipette introduced into the vagina, will show the presence of numerous, large, cornified, non-nucleated cells. The presence of these cells represents the estrous phase of the sexual cycle and serves as a positive criteria for the estrogenic activity of the substance injected.

Numerous variations in the original procedure have been developed using either adult ovariectomized rats or mice (31-35) in attempts to eliminate variables and to increase the sensitivity of the test animal.

Other biological assay techniques have been described in the literature which are dependent upon the effect of the estrogenic hormones on both primary and secondary sexual characteristics of the test animals (36-41). Of these the most commonly used is the technique involving the effect of estrogens on the uterine weight of the test animal (38).

In the Allen and Doisy bioassay method (1) the potency of the estrogenic substance was expressed in units. This unit was defined as the minimum amount necessary to induce estrous with complete cornification of the vaginal mucosa, as judged

from a smear, in 75% of a large group of ovariectomized, sexually mature rats. Other workers established a unit based on the effect on the mouse (31). Efforts to correlate data of different investigators and attempts to find a constant factor for converting the rat unit to the mouse unit met repeated failure. Investigation has shown that the route by which the assayed substance was administered to the test animal, as well as the solvent, number of injections, the interval between injections and the sensitivity of the animal to the material, were all factors influencing the estrous response (42).

A definite advance was made when the International Unit was introduced by the Conference on the Standardization of the Sex Hormones of the League of Nations Health Organization in 1933 (43). The standard selected was 0.1 gamma of estrone which was to be considered as one unit. The assay of new estrogenic substance was to be carried out on some test animal, as the rat or mouse. The standard and the unknown were to be administered simultaneously and under identical conditions to two series of test animals and the strength of the unknown was to be expressed in terms of estrone.

The International Conference stated that the International Unit was approximately one-third of the Allen and Doisy rat unit (1), but other workers have placed the value at from one-fifth to one-thirty-eighth of the rat unit (44). Investigators state that the variation in the rat unit or the mouse unit may be as great as 1,000 percent (31).

An International Benzoate Unit has also been established, equivalent to 0.1 gamma of estradiol benzoate and is known as one Benzoate Unit.

(2) Chemical Assay Methods

Since the elucidation of the chemical structure of the estrogenic hormones, many attempts have been made to replace the biological assay of these hormones by a simple chemical method of estimation.

Marrian (45) is of the opinion that the only important colorimetric reactions for the assay of urinary estrogens are those based on the Kober reaction (46).

Kober based his assay procedure on the observation that the green fluorescence resulting from the treatment of pure estrone with concentrated sulfuric acid was changed to red upon dilution with water. This red colour was intensified by the presence of phenol. He was able to compare these colours with standard solutions of cresol red and to determine the amount of estrogenic substance present. Kober observed that the colour developed with the pure substance could be completely removed by warming with three percent hydrogen peroxide. It was assumed, therefore, that any colour remaining was due to substances other than estrogens and this colour was subtracted from the maximum red value after making necessary volume corrections. This reaction is highly specific to the natural estrogens and, when carried out under certain conditions, pink colours of equal intensity are developed by estrone, estriol and estradiol. Improvements in the technique of carrying out the colour reactions have been made by Cohen and Marrian (17), Venning et al (19) and Bachman (47) amongst others. Various modifications in the composition of the reagent have also been made, the most important being the substitution of beta-napthol for phenol (48).

The presence of the phenolic group in ring A of the estrogenic hormones and their resultant ability to be converted into azo dyes, has been made the basis of quantitative colorimetric reactions by some investigators (49-52).

The diazo reaction of estriol was first noted by Harrington and Schupbach (49) who used para-nitroaniline with aqueous solutions. They obtained solutions having a yellow colour which was difficult to match and did not conform strictly to Beer's Law.

Schmulovitz and Wylie (50) showed that estrone could be estimated much more readily by coupling it with diazobenzene sulfonic acid to give an azo dye. Beta-napthol was used as the colour standard and comparison was made in a Duboscq colorimeter at the end of five minutes. A red colour which was found to be permanent for one hour was produced by the addition of sodium hydroxide.

The diazo method of Talbot (51) involves the coupling of the phenolic hydroxyl with diazotized dianisidine. Talbot suggested the test as being specific for the weakly phenolic ketone, estrone. This claim was later investigated by Riefenstein and Dempsey (52) who found the method to be accurate within limits and substantiated that the procedure was satisfactory only for the estimation of estrone.

Other reactions have also been described in the literature which are specific for one or more of the estrogenic hormones to be found in human urine.

Veitch and Malone (53) prepared the 2, 4 -dinitrophenylhydrazone of estrone and the high tinctorial power of the resulting solution suggested its use for the quantitative

estimation of this estrogen. They found that the alcoholic solution exhibited maximum absorption in the ultra violet region at 440 mu which was out of the range of most photo-electric colorimeters and thus necessitated spectroscopic analysis. The method has not been adapted to the assay of biological extracts.

David (54) has described the development of a blue colour resulting from the treatment of pure estriol crystals with concentrated sulfuric acid followed by arsenic acid. The test is specific for estriol, but Pincus et al (55) found that the test was only half as sensitive as the phenolsulfonic acid test (46) and could not be applied to the assay of estriol in urinary extracts because of the bluish cloudy suspension which formed.

Bachman (47) has also described a specific colour reaction for estriol. The method consists of heating the estrogen at 150° C. with sodium para-phenolsulfonate and phosphoric acid. A stable violet-pink colour is produced which is examined in the Evelyn photoelectric colorimeter. The reaction can be used to determine the amount of estriol in a mixture of estrone and estradiol. The pink colour is highly specific for estriol and the other estrogenic hormones give no evidence of the pink colour.

Szego and Samuels (56) substituted guaiacolsulfonic acid for phenolsulfonic acid in the Kober reaction (46) and obtained a pink colour with estrone having a similiar intensity to that produced with phenolsulfonic acid. The claim is made that the results are more readily reproducible than with the Kober reagent and that, using the conditions specified, the reaction is specific for estrone.

An assay procedure described by Pincus et al (55) involves the reaction of benzoyl chloride with estrone in the presence of zinc chloride and glacial acetic acid. In this method a new curve must be prepared for every determination, but sensitivity is claimed to be equal to that of the phenolsulfonic acid procedure (46).

Every colorimetric procedure appears to lack specificity when adapted to the assay of urinary extracts.(45). Attempts to assay pregnacy urine utilizing the Kober (46) reaction have given readings as great as four times the amount of hormone actually present. This is due to non-specific materials which also give a colour change with this test (19). Cohen and Marrian (16), on the other hand, state that the test is not applicable to the urine of non-pregnant individuals. The methods of Stimmel (27) and of Bachman and Petit (21) are fairly satisfactory for excretions of one to three milligrams daily. In the absence of pregnacy, however, the average daily excretion is less than 100 micrograms daily (57).

Friedgood and Garst (58) have applied ultraviolet absorption to the assay of minute amounts of estrogenic hormones, and others (59) have developed a polarographic technique. However, in these procedures, also, difficulty has been encountered in application to the assay of urinary extracts.

More recently Jailer (60, 61) has reported the successful assay of non-pregnant urines by fluorescence. The extraction procedure of Bachman and Petit (21) is used to obtain the concentrates and the non-specific fluorescence is corrected for by means of an equation similiar to that employed by Stimmel (27).

Finklestein et al (62) and Bates and Cohen (63) have also

described fluorescent techniques for the estimation of estrogens. The former workers have used phosphoric acid to develope fluorescence while the latter have employed sulfuric acid. Neither of these procedures have been adapted to the estimation of the estrogenic content of urinary extracts.

THE PROBLEM

THE PHOTOFLUOROMETRIC ESTIMATION OF ESTROGENS AND ANDROGENS

In the colorimetric assay technique of Kober (48) the addition of sulfuric acid to estrone causes a green fluorescence to be produced. Prior to the commencement of our examination very little attention had been paid to this fluorescence. Sala (64) had previously employed this phenomenon as a qualitative test for pregnacy, and Cuboni (65) to determine the presence of estrone in the urine of pregnant mares. However, during the time we have been conducting our investigation, several papers have been published (60-63) involving the quantitative analysis of the estrogens by fluorescent estimation. The preliminary reports of two of these were so vague as to be impossible to check and it was necessary to wait until detailed reports were forthcoming to be able to evaluate them. We have been able, however, in the light of information received from these reports, to make improvements in our earlier procedures,

The purpose of our investigation was to attempt the control of this fluorescence in such a manner as to develop a quantitative assay technique. We hoped by so doing to devise a procedure which would have advantages over the existing methods of assay for estrogens as regards sensitivity, accuracy, simplicity, and specificity.

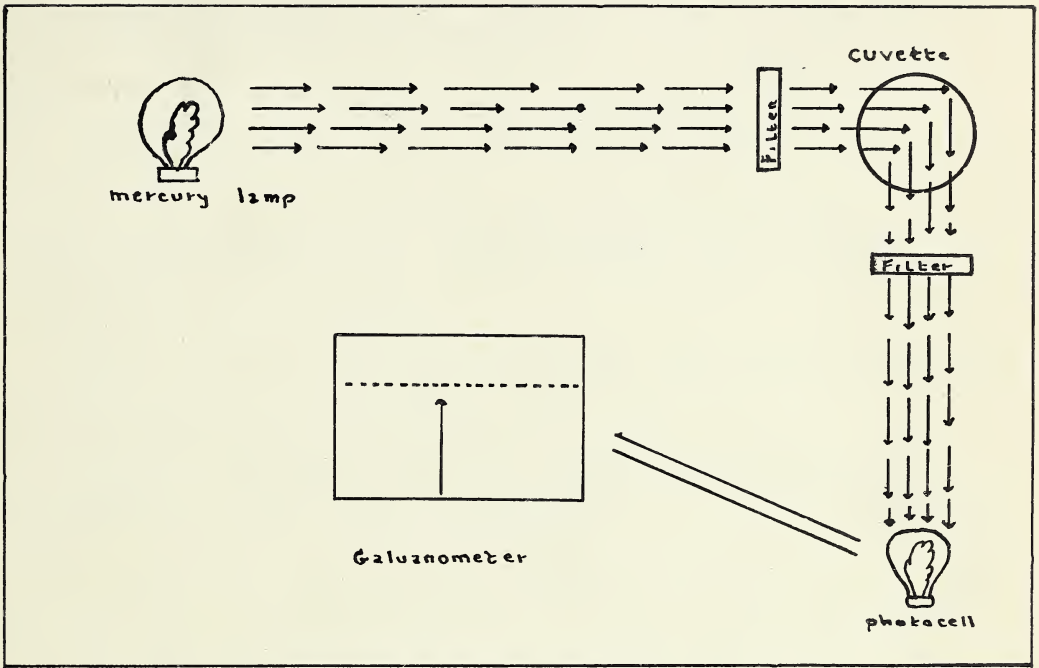
The similiar chemical nature of the androgens suggested the possibility that fluorescence might also be produced by these compounds which could be adapted for assay procedures.

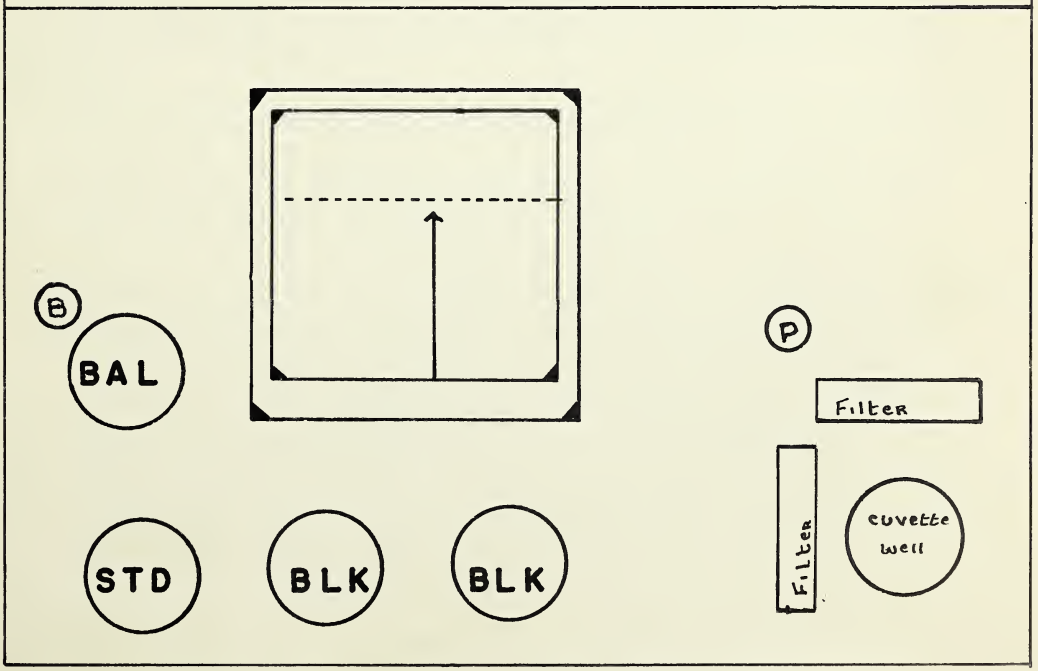
PROCEDURE

FIGURE 1

COLEMAN PHOTOFLUOROMETER

MODEL 12





APPARATUS

The apparatus employed for our determinations was the Coleman Photofluorometer, Model 12 (Figure 1). The extreme fluctuations in the line voltage supplied to our laboratory, and the resultant effect on lamp sensitivity, caused us to employ a Variac so as to be able to note these voltage changes and thus make the necessary adjustments. For the Model 12 Photofluorometer the Variac is set to read 115 volts.

Before running any tests with this instrument it is essential that both its mercury vapor lamp and electronic amplifier reach equilibrium. The procedure for the attainment of equilibrium is as follows:

1. Insert in panel opening number one, the proper filter for the fluorescent measurement involved.
2. In panel opening number two, insert the corresponding filter to match filter number one.
3. Energize the instrument by rotating the "STD" knob clockwise until it just clicks which turns on the amplifier and also starts the fan and excites the mercury vapor lamp.
4. After thirty seconds, continue rotation of the "STD" knob to the extreme clockwise position, thus adjusting the amplifier to the condition of maximum sensitivity.
5. Depress the button "B" and, while in the depressed position, adjust knob "BAL" until the instrument reads zero. The purpose of this adjustment is to balance the electronic amplifier to the condition of the "A" batteries.
6. Adjust the "BLK" (blank) knob until the instrument meter reads zero. This knob serves to balance the amplifier to

zero and may subsquently be used to correct for the fluorescence of blanks during the actual analysis of fluorescent samples.

7. Allow at least ten minutes for the instrument to stabilize.

8. After stability is attained, repeat adjustment 5 and then 6.

It is then necessary to adjust the amplifier sensitivity. The instrument sensitivity is dependent, in part, on the intensity of the mercury vapor lamp, which in turn depends on the voltage and frequency of the current supply. Since these latter factors may vary, we have found that it is essential to check the sensitivity of the Electronic Photofluorometer immediately before each test, by inserting in the instrument a cuvette containing a standard reference solution. The "STD" knob may then be adjusted until the meter reads a value selected as representing the proper sensitivity of the lamp for the determination under consideration.

For the actual calibration of the instrument or the determination of an unknown quantity of fluorescent material the procedure is as follows:

1. Complete adjustments 1 through 8 as previously outlined.

2. Place the cuvette of standard reference solution in the cuvette well, cover with the cap, and then open the lamp shutter by fully depressing button "P". With "P" fully depressed, adjust the "STD" knob until the meter reads a value consistent with the concentration of the sample to be determined.

3. Insert a cuvette containing the "blank" solution, cover, again depress button "P" and with "P" again fully depressed adjust the meter to read zero.

4. Next insert a cuvette containing a solution of the material to be tested, cover, fully depress button "P" and note the reading.

The adjustment of the sensitivity by means of the standard reference solution must be repeated before every determination. However, this adjustment with the "STD" knob must not be made again until the "BLK" knob has been returned to its original position; that is, with the meter reading zero when "P" is in the released position with the light shutter closed.

The extreme sensitivity of this apparatus demands a constant examination of the mercury vapor lamp, amplifier tube and the batteries to maintain the instrument operating at the peak of efficiency. All these parts should be examined periodically for signs of failure. Any deficiency in these parts affects the readings and falsifies results.

SELECTION OF THE FILTERS

In photometric analysis a filter is used to isolate as narrow a band of light as is feasible, and this band must be centered as closely as possible to the point of minimum transmittancy of the sample.

With the Electronic Photofluorometer it is necessary to use two filters. The first of these filters, either B-1 or B-2 is placed between the mercury vapor lamp and the cuvette containing the reaction mixture. These two filters pass different light bands, permitting the rays to strike the cuvette when "P" is depressed. Filter B-1 passes the 365 mu band and filter B-2 the 436 mu band of mercury.

The second filter is placed between the cuvette and the phototube. The purpose of this second filter is to screen

out the rays from the wave band passed by the corresponding primary filter, while allowing the fluorescent rays from the sample to enter the phototube and be measured. With primary filter B-1 the manufacturers suggest using secondary filter PC-1 and with filter B-2, secondary filter PC-2.

Preliminary experiments with the filters showed that a much greater sensitivity, with our solutions, was obtained with the B-1, PC-1 combination than with the B-2, PC-2 combination. These filters were therefore used throughout. This combination was also used by Bates and Cohen (63) in their procedure for the fluorescent analysis of estrogenic hormones.

Jailer (60,64) has since reported the use of still another secondary filter PC-9A. This filter was used in combination with both B-1 and B-2 primary filters. A report on our findings with these filter combinations will be presented later in this paper.

THE STANDARD REFERENCE SOLUTION

For the purpose of a standard reference solution we selected a solution of quinine sulfate in dilute sulfuric acid as recommended by the Coleman Company. This solution had previously been used in the fluorometric analysis of thiamin solutions and was recommended because of its photochemical stability.

Investigation of different strength solutions of quinine sulfate in sulfuric acid caused us to select a strength of 0.1 microgram per millilitre of quinine sulfate in 0.1 N sulfuric acid. Selection of this strength was made as follows:

One milligram of quinine sulfate was dissolved in 100 millilitres of 0.1 N sulfuric acid. Various dilutions of this

solution were made in 0.1 N sulfuric acid and examined in the instrument. The strength selected was the one enabling the "STD" knob to be rotated so as to make the meter read 100. With stronger concentrations it was found impossible to adjust the reading to 100 or below, as the fluorescent intensity was too great.

One milligram of quinine sulfate is dissolved in 100 millilitres of 0.1 N sulfuric acid and stored under refrigeration. One millilitre of this solution is diluted to 100 millilitres in a volumetric flask with 0.1 N sulfuric acid. This dilution is prepared fresh daily as it has been noticed that the weaker solution has a tendency to decrease in fluorescent intensity if allowed to stand over several days. Eight millilitre aliquots of this latter solution are used for standardization.

THE " BLANK "

The solvent and other chemicals used for preparing the reaction mixture exhibit some fluorescence in themselves and the activity of these "blank" constitutents must be considered in all measurements made.

The "blank" solution is prepared by combining in the same manner and amounts all the ingredients of the corresponding sample solution except the estrogen.

With the Electronic Photofluorometer the "blank" reading is subtracted electrically by inserting a cuvette of this solution, depressing "P" and then adjusting the "BLK" knob until the meter reads zero. When the corresponding sample is then inserted and "P" again depressed, the reading obtained is due to the fluorescence produced by the estrogen being examined.

REAGENTS AND EQUIPMENT

95% sulfuric acid C.P. (British Drug Houses) was employed in all our determinations. All other solvents employed in our determinations were either redistilled or were of unquestionable purity. The only solvent we believed to be in the latter classification was anaesthetic ether, which was employed in extraction procedures.

All solutions were prepared using volumetric apparatus; and micro-burettes were employed for minute measurements. No stop-cock lubricant was used since it might give rise to extraneous fluorescence.

EXPERIMENTAL

SECTION ONE

The development of any analytical procedure requires an investigation and standardization of any variables which effect the reaction. Our initial investigation, therefore, was aimed at a determination of the variables which might be involved in the production of the fluorescence.

For our experimental work we selected crystalline estrone as the test material. For test purposes we initially made up a solution of 10 milligrams of crystalline estrone in 100 millilitres of 95% ethenol. Various aliquots and dilutions of this solution were employed in the different procedures undertaken.

We have expressed the concentrations of the several estrogenic solutions tested in terms of micrograms of the active substance being investigated. This procedure has been employed in all tables, graphs and charts.

The readings designated refer to readings of the galvanometer and are not meant to imply any special unitage. A reading of 100 indicates that the reading obtained was beyond the galvanometer range.

In Table One we have presented the results of our investigation to find the approximate range over which estrone would display fluorescence. This investigation was performed by adding one millilitre of 95% sulfuric acid to the estrone contained in one millilitre of 95% ethanol and diluting the mixture with 95% ethanol to provide the eight millilitres necessary for measurement. The resultant solution was tested for fluorescence immediately.

From this table it is evident that the range which might

be determined with the Electronic Photofluorometer would appear to be from 25 micrograms to 75 micrograms. The extreme variation and lack of consistency of readings indicated that the production of the fluorescence would require careful control to achieve uniformity of results.

TABLE ONE

PRODUCTION OF FLUORESCENCE BY CRYSTALLINE ESTRONE

Estrone Micrograms	Galvanometer Readings		
1000	100+	100	100
500	100+	100	100
300	100	100	100
200	100	100	100
100	100	100	100
75	31	48	27
50	100	53	76
40	4	16	48
30	23	11	43
25	3	14	9
20	1	0	0

Since it was thought possible that part of the variation might be due to the strength of acid employed, several dilutions were tested using the same procedure. The results are recorded in Table Two.

While the use of weaker solutions of sulfuric acid did not overcome the variances obtained previously with concentrated

TABLE TWO

EFFECT OF DILUTE SOLUTIONS OF SULFURIC ACID ON CRYSTALLINE ESTRONE IN 95% ETHANOL

Estrone Micrograms	Acid % v/v	Galvanometer Readings	
30	50	100	100
30	30	100	100
30	25	100	100
30	20	100	100
30	15	100	100
30	10	100	100
30	5	100	100
30	2	100	100
30	1	100	100
30	0.5	100	100
30	0.25	100	100
30	0.20	100	100
20	0.20	8.5	6
10	0.20	0	2
5	0.20	0	3
2.5	0.20	20	50
2.0	0.20	24	13

sulfuric acid, an interesting fact was revealed by this series of tests. This was the increase in the intensity of fluorescence produced by the use of the weaker acid. In our initial investigation concentrations of 30 micrograms gave a reading of 43 as their maximum, whereas in this series the same concentration gave readings of 100 in every instance. Evidence of this increased sensitivity was also shown in that we were now able to obtain results with concentrations as low as two micrograms.

As the use of weaker acid solutions did not achieve uniform results we decided to employ several other solvents in place of 95% ethanol. Accordingly, 10 milligram percent solutions of estrone were made up in n-butanol, ether, and absolute ethanol. The estrone contained in seven millilitres of the solvent being investigated was added to one millilitre of the acid and tested for fluorescence immediately. In some cases the acid was diluted with water, in others with the solvent being investigated.

In Table Three are recorded the results obtained with the three different solvents as well as the results where the solvent had been evaporated off leaving the dried estrone residue.

When the sulfuric acid was added in aqueous dilution to the estrone dissolved in n-butanol the immiscibility of the two liquids produced a cloudiness which reduced the intensity. When the acid was added diluted with n-butanol the 0.2% gave a range of only 9 on the galvanometer for 10 micrograms to 300 micrograms of estrone. The 1% gave a wider range but agreement and uniformity were poor. The sensitivity with

ether was very poor, requiring 100 to 200 micrograms to produce a significant reading. With absolute ethanol there was some improvement in relation of degree of fluorescence to amount of estrone. When 95% sulfuric acid was added to aliquots of dried estrone all the readings were above 100.

The high sensitivity when concentrated sulfuric acid was added to dried estrone suggested the possibility that a smaller amount of acid might give an amount of fluorescence within the range of the galvanometer. Table Four shows the results obtained when 1 to 8 millilitres of 95% sulfuric acid were added to 100 micrograms of estrone. Where necessary the solution was diluted to eight millilitres with absolute ethanol.

TABLE THREE

EFFECT OF SOLVENT ON DEVELOPMENT OF FLUORESCENCE OF ESTRONE						
Estrone	N-Butanol			Ether	Absolute Ethanol	No Solvent
Concentration in Micrograms	0.2% V/V Sulfuric Acid in Water	0.2% V/V Sulfuric Acid in N-Butanol	1.0% V/V Sulfuric Acid in N-Butanol	1.0% V/V Sulfuric Acid in Ether	1.0% V/V Sulfuric Acid in Ethanol	95% Sulfuric Acid
300	38 38 38	36 36 36	32 32	38 35	25 30 32	59 100
250	13 14 16	28 28 28	27 30	6 8	25 27 30	100 100
200	10 15 13	19 20 20	23 24	4 4	12 19 21	100 100
150	10 15 14	39 39 39	13 22	1 1	13 14 15	100 100
100	6 6 6	32 32 32	8 8	0 1	11 13 13	100 100
50	5 4 3	25 25 25	0 4	0 0	4 5 7	100 100
40	3 3 2	27 27 27	8 6	- -	3 4 5	100 100
30	0 5 3	26 26 26	2 5	- -	2 4 4	100 100
20	0 3 2	27 27 28	2 2	- -	3 3 4	100 100
10	2 4 4	27 27 27	4 4	- -	0 1 2	100 100

TABLE FOUR

EFFECT OF ACID ON FLUORESCENCE OF ESTRONE				
Estrone-Micrograms	Mls. of 95% sulfuric acid	Galvanometer Readings		
100	8	100+	100+	100+
100	7	100+	100+	100+
100	6	100+	100+	100+
100	5	92	88	90
100	4	100+	97	96
100	3	86	95	95
100	2	98	99	98
100	1	95	93	94

TABLE FIVE

FLUORESCENCE PRODUCED WITH ONE MILLILITRE OF 95% SULFURIC ACID				
Estrone-Micrograms	Galvanometer Readings			
100	98	100+	100+	100+
90	100+	100+	100+	100+
80	95	100+	100+	97
70	60	97	83	92
60	89	70	45	84
50	86	74	77	74
40	66	52	66	55
30	59	43	22	40
20	27	29	15	15
10	8	5	10	7

Table Five presents the results obtained when one millilitre of 95% acid was added to different quantities of dried estrone. In each case dilution to eight millilitres was made with absolute ethanol. Since this technique appeared to hold promise, further work was undertaken using the procedure of adding acid to dried estrone. Estrone was dissolved in absolute ethanol and aliquots evaporated to dryness.

The addition of the absolute ethanol to the sulfuric acid produced an elevated temperature. Our practice had been to test this solution for fluorescence without delay on completion of mixing. During the course of our investigations, however, evidence had been accumulating as to the effect of heat on the final reading obtained. As the solution cooled to room temperature the intensity of fluorescence appeared to decrease to a certain point where the readings would remain constant. It was thought possible that the variance in the readings might be

TABLE SIX

EFFECT OF TEMPERATURE ON READINGS			
Series One		Series Two	
Temp.	Reading	Temp.	Reading
92°	61	83°	72
81°	37	70°	45
50°	33	48°	32
28°	31	31°	31
27°	29	26°	29
25°	29	25°	29
15°	29	15°	29

due to differences in temperature at the time readings were made. Table Six shows that marked variations are to be noted above 27° C., but that below that temperature readings were consistent. As a result we have since employed the practice of

cooling the reaction mixture under the cold water tap to 25° C.

An investigation of the time required for the reaction to go to completion showed that with concentrations of estrone from 5 micrograms to 80 micrograms the readings could be increased to 100+ by allowing the estrone to stand for thirty minutes in the presence of one millilitre of 95% sulfuric acid before adding the absolute ethanol to eight millilitres. From this series of results we were inclined to believe that perhaps some of our former discrepancies had been due to incomplete development of the fluorescence. By referring to Table Five it may be seen that with a concentration of 70 micrograms, for example, the readings obtained varied from 60 to 93. It was thought that this extreme variance could have been caused by different periods of time elapsing before the mixture was tested for fluorescence. However, in Table Six where we have recorded the effects of the temperature of the reaction mixture on the reading, we obtained good agreement of results in the two series investigated. These series of readings were obtained, however, by allowing the reaction mixture to cool slowly to the temperature stated. It seemed possible that the period of time elapsing between the first and last reading had helped to contribute to the reproducibility obtained.

The increase in fluorescent intensity on standing prompted an attempt to use heat to bring the reaction to completion more quickly. A temperature of 120° C. was arbitrarily chosen and using the same concentration range as above, the samples were heated in an oil bath with occasional agitation. All concentrations tested gave a reading of 100+.

An attempt was made to dilute the solutions to a point where

the intensity would be within the range of the galvanometer. Dilution of the mixture to 100 millilitres with distilled water produced a suitable intensity and the results obtained with the several different series were fairly uniform, (Table Seven)

Utilizing this dilution, a closer investigation of the reaction temperature, the time required for the completion of reaction, and the effect of agitation during the heating period was conducted. Results showed (Tables Eight and Nine) that our selection of 120° C. for a period of twenty minutes as reaction temperature and time had been fortunate as these appeared to be suitable for complete reaction. Shorter reaction periods or lower temperatures decreased the intensity of fluorescence while higher temperatures or longer periods for reaction did not have the effect of producing a greater intensity. Continuous agitation during the heating period appeared to increase slightly the results obtained and to contribute to reproducibility.

The apparatus used for agitation purposes consisted of a wire basket in which the reaction tubes were set immersed in an oil bath. This basket was moved gently back and forth by means of a wire attachment to an eccentric arm of an electric motor.

From the foregoing investigations a procedure was developed for the assay of estrone, and also applied to the assay of estradiol and estradiol benzoate.

Technique One

An aliquot of an alcoholic solution of estrone is evaporated to dryness in vacuo on a boiling water bath. One millilitre of 95% sulfuric acid is added and the mixture is heated at 120° C. for twenty minutes with agitation. The mixture is cooled

TABLE SEVEN

REPRODUCIBILITY OF RESULTS ON DILUTION TO 100 MILLILITRES WITH WATER					
Micrograms of Estrone	Galvanometer Readings				
20	35	37	35	34	34
30	43	45	43	41	40
40	49	55	50	51	50
50	58	64	64	63	64

TABLE EIGHT

PERIOD OF HEATING REQUIRED AT 120°C.					
30 MICROGRAMS OF ESTRONE TESTED					
Time in Minutes	Galvanometer Readings				
10	24	23	20	26	18
20	42	43	43	43	42
40	43	43	42	42	43
60	46	44	43	44	40

TABLE NINE

TEMPERATURE OF HEATING AND EFFECT OF AGITATION OVER A TWENTY MINUTE PERIOD						
30 MICROGRAMS OF ESTRONE TESTED						
Temp. in Degrees C.	AGITATION			NO AGITATION		
	Galvanometer Readings ——					
100°	31	37	33	21	29	26
120°	42	43	43	25	32	33
140°	43	43	43	33	36	40

immediately under the cold water tap to a temperature of 25°C. or lower and diluted to 100 millilitres with distilled water in a volumetric flask. An aliquot of this solution, consisting of at least eight millilitres, is tested for fluorescence. A blank is run with all determinations; and a reference standard of 0.001 milligrams % quinine sulfate in 0.1 N sulfuric acid is employed. The "STD" knob is set at 100 using this standard reference solution immediately preceeding every determination.

For reaction tubes we employed Pyrex test tubes with a diameter of 25 mm. and approximately 8 to 10 cms. in height. The hormone solution was evaporated to dryness in these test tubes and the 95% sulfuric acid was added directly to the dried residue and then heated.

In Tables Ten to Twelve we have shown the results of a series of determinations carried out with estrone, estradiol and estradiol benzoate utilizing the procedure outlined. Plotting the results of intensity of fluorescence against concentration we were able to show that a linear relationship existed between these two (Graph One). The curve obtained with estradiol does not quite split the axis at zero; the point of intersection is such that a concentration of two micrograms would give a reading of zero. Estrone and estradiol benzoate, however, follow Beer's Law.

The intensity of fluorescence produced by estradiol is approximately 12% less than that shown by estrone. Estradiol benzoate in turn shows approximately 31% less fluorescence than estradiol. The calculations are computed at a concentration of 50 micrograms of the hormones. With estrone a range of 10 to 65 micrograms was covered, whereas with estradiol and

TABLE TEN

FLUOROMETRIC ESTIMATION OF CRYSTALLINE ESTRONE BY TECHNIQUE ONE

Micrograms of estrone	Galvanometer Readings										
10	16.0	14.0	14.0	15.0	14.0	15.0	13.0				
15	19.0	24.0	23.0	21.0	25.0	26.0					
20	30.0	31.0	33.0	32.5	30.0	25.0	31.0	34.0			
25	35.0	38.0	37.0	42.0	39.0	38.0	41.0	41.0			
30	45.0	48.0	44.0	44.0	42.0	40.0	44.0	45.0	44.0		
35	50.0	54.0	53.0	52.0	54.0	54.0	53.0				
40	53.0	58.0	58.0	60.0	59.0	60.0	61.0				
45	68.0	67.0	69.0	67.0	66.0						
50	70.0	76.0	74.0	70.0	76.0	74.0					
55	75.0	82.0	67.0	81.0	74.0	80.0	66.0	78.0	79.0	81.0	
60	89.0	69.0	80.0	83.0	86.0	89.0	92.0	80.0	80.0	88.0	96.0
65	100.0+	97.0	100.0+	99.0	96.0	91.0	99.0	99.0	93.0	95.0	

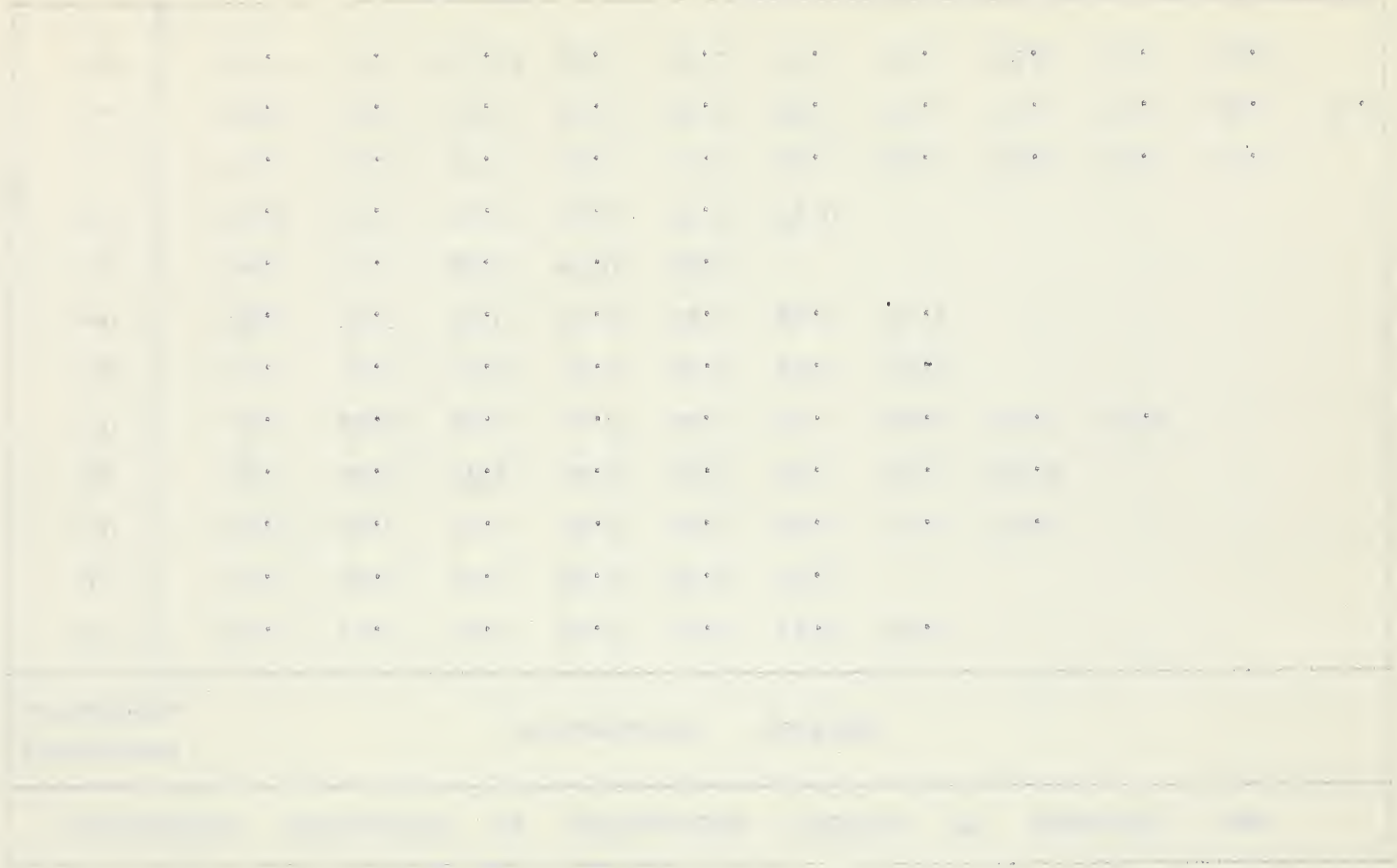


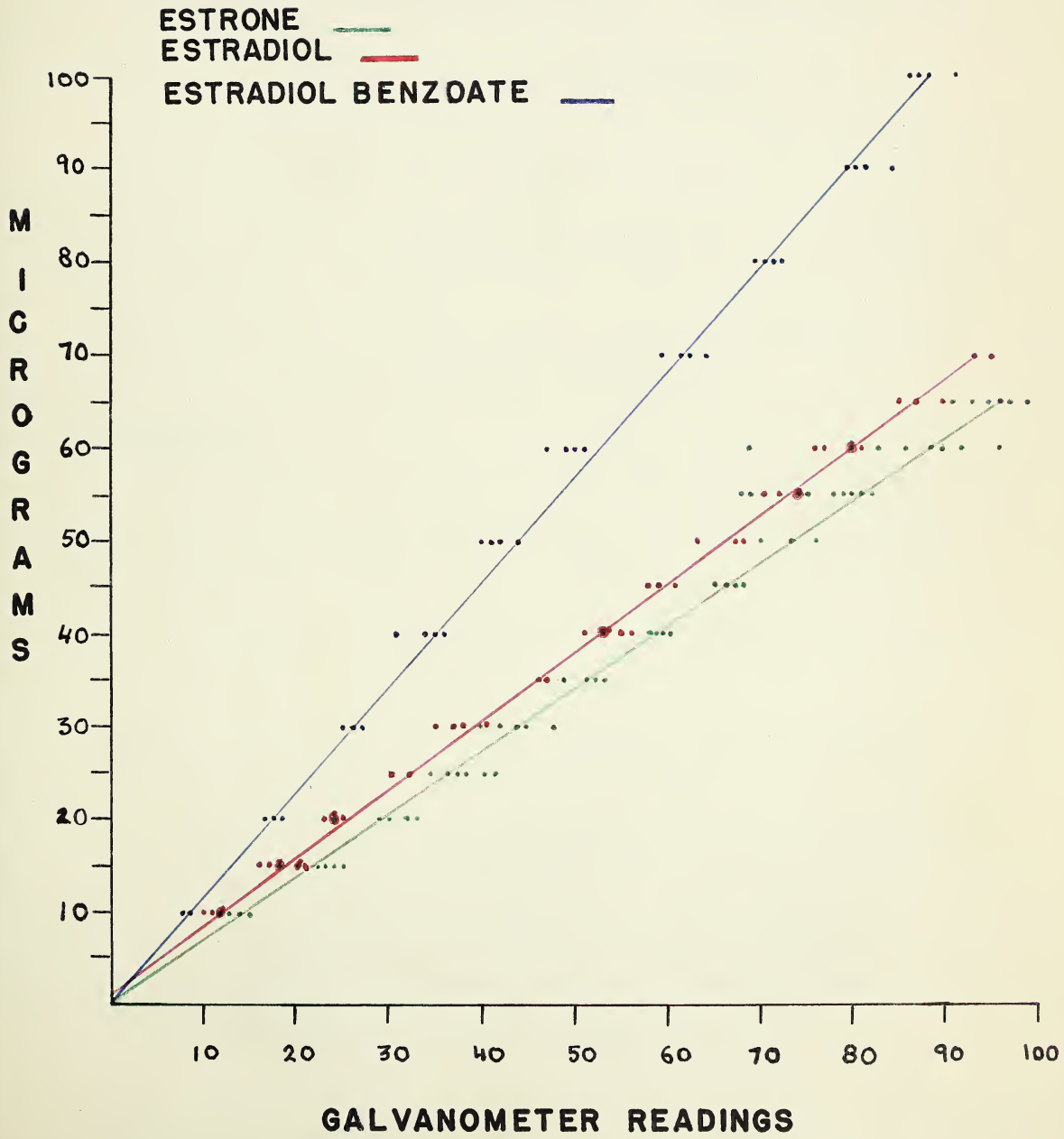
TABLE ELEVEN

FLUOROMETRIC ESTIMATION OF ESTRADIOL BY TECHNIQUE ONE					
Micrograms of estradiol	Galvanometer Readings				
10	11.0	10.0	10.0	12.0	11.0
15	17.0	20.0	16.0	18.0	21.0
20	24.0	23.0	24.0	25.0	25.0
25	30.0	32.0	30.0	32.0	32.0
30	35.0	35.0	37.0	40.0	38.0
35	47.0	46.0	47.0	46.0	47.0
40	51.0	53.0	55.0	56.0	53.0
45	59.0	58.0	58.0	61.0	59.0
50	67.0	68.0	67.0	63.0	67.0
55	72.0	71.5	70.0	74.0	74.0
60	80.0	81.0	77.0	76.0	80.0
65	87.0	90.0	87.0	87.0	85.0
70	93.0	93.0	93.0	95.0	93.0
75	100.0+	99.0	100.0+	100.0+	100.0+

TABLE TWELVE

FLUOROMETRIC ESTIMATION OF CRYSTALLINE ESTRADIOL BENZOATE BY TECHNIQUE ONE					
Micrograms of Estradiol Benzoate	Galvanometer Readings				
10	8.0	9.0	8.0	9.0	9.0
20	18.0	17.0	19.0	17.0	19.0
30	25.0	24.0	25.0	25.0	26.0
40	31.0	34.0	35.0	36.0	34.0
50	41.0	40.0	44.0	44.0	42.0
60	51.0	51.0	47.0	49.0	50.0
70	61.0	59.0	64.0	62.0	61.0
80	71.0	72.0	69.0	70.0	70.0
90	79.0	81.0	79.0	84.0	80.0
100	88.0	87.0	87.0	91.0	86.0

GRAPH I


ESTRONE
ESTRADIOL
ESTRADIOL BENZOATE
MICROGRAMS
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
100
GALVANOMETER READINGS

estradiol benzoate the ranges which could be determined were 10 to 70 micrograms and 10 to 100 micrograms respectively.

While the sensitivity and accuracy of this method were not quite as satisfactory as one would like, this technique served to demonstrate that the fluorescence produced by estrogens in the presence of sulfuric acid could be controlled in such a manner as to provide a means of quantitative assay.

PROGESTERONE AND THE ANDROGENS

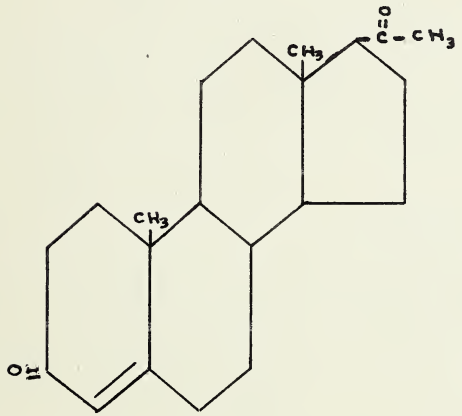
The similiarity in chemical structure of the progesterone group of hormones and of the male sex hormones to that of the estrogenic hormones suggested the possibility of utilizing the above procedure for the estimation of these compounds. These substances are also derivatives of cyclopentanophenanthrene as are the estrogenic hormones (Chart Two).

Progesterone

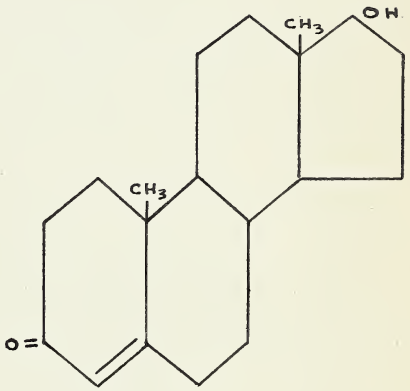
Progesterone gave approximately the same readings as those obtained with similiar concentrations of crystalline estrone. However, several factors made us doubt that these readings were entirely reliable. Fluorescence did not appear on the addition of the 95% sulfuric acid to the progesterone but after heating for twenty minutes at 120° C. a brown colouration appeared. The fluorescence produced by estrone and estradiol was a clear bright yellow whereas with progesterone the colour exhibited showed every evidence of being caused by charring. The readings obtained with equal concentrations showed no similarity and we therefore concluded that the procedure was of no value for the assay of progesterone (Table Thirteen).

CHART TWO

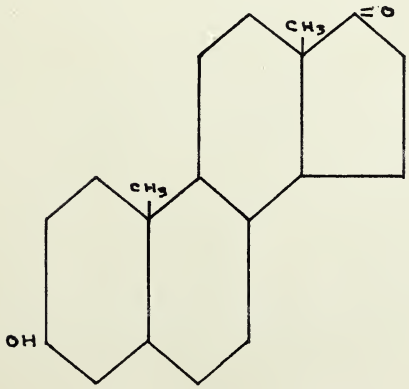
STRUCTURAL FORMULAS OF PROGESTERONE AND THE ANDROGENS



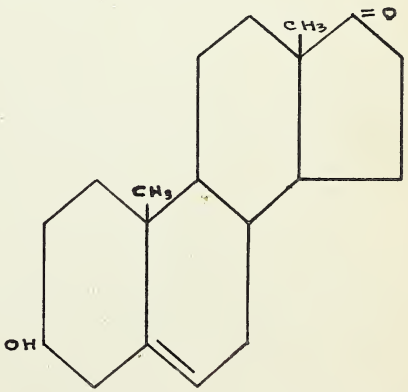
PROGESTERONE



TESTOSTERONE



ANDROSTERONE



DEHYDROANDROSTERONE

TABLE THIRTEEN

FLUORESCENCE OF PROGESTERONE IN SULFURIC ACID					
Estrone Micrograms	Galvanometer Readings				
10	17.0	13.0	11.0	11.5	8.0
20	33.0	14.0	28.0	25.5	37.0
30	51.5	41.5	54.5	37.0	43.0
40	55.5	74.5	67.0	82.0	62.0

Androgens

The results obtained with testosterone were also unsatisfactory. There was evidence of charring and the results obtained with equal concentrations of hormone varied greatly (Table Fourteen).

TABLE FOURTEEN

FLUORESCENCE OF TESTOSTERONE IN SULFURIC ACID				
Testosterone Micrograms	Galvanometer Readings			
10	25.0	21.0	73.0	64.0
15	47.5	78.0	100+	39.5
20	63.5	37.0	100+	86.0
25	100+	100+	100+	100+
30	100+	100+	100+	100+

An insufficient supply of crystalline testosterone forced us to employ androsterone for further investigations of the androgenic hormones. Following the procedure as previously outlined we again obtained very irregular results.

As it was apparent that the method developed for estrogens was not suitable for androgens, an investigation of the variables as they apply to androsterone was undertaken. One complication

in the case of androsterone was its volatility (69)

In Tables Fifteen to Eighteen we have compiled the results of a series of experiments undertaken to determine the effect on the developed fluorescence of drying in vacuo, the heating period and the amount of acid. From these investigations the following observations were made.

1. The evaporation in vacuo on a boiling water bath of an alcoholic solution of androsterone resulted in readings which varied greatly from series to series for results run in duplicate. The evaporation for longer periods than the fifteen minutes which had been employed for the estrogens did not overcome this variance. The evaporation in vacuo at a lower temperature (75°C) gave much better reproducibility.
2. Heating at a lower temperature during the reaction period produced better agreement between the results run in duplicate but these results could not be reproduced in a second series.
3. Allowing the reaction to go to completion without the aid of heat and not evaporating the alcoholic solution to dryness produced fluorescence of similiar intensity in equal concentrations.
4. The readings obtained with solutions allowed to stand in the dark were not markedly different from the readings obtained with solutions allowed to react in daylight.
5. The addition of one millilitre of 95% sulfuric acid to an alcoholic solution of androsterone produced similiar readings with several different concentrations ruling out the possibility of a linear relationship existing between intensity and concentration when this technique was

employed.

6. The addition of an amount of acid equal to the volume of alcoholic solution of androsterone being examined improved sensitivity.

From these observations we were able to develop the following procedure which was used to determine the concentrations of androsterone, dehydroandrosterone and dehydroandrosterone acetate in solution in absolute ethanol.

To an aliquot of an alcoholic solution of androgenic hormone is added an equal volume of 95% sulfuric acid. The two solutions are thoroughly mixed and allowed to react in a stoppered container for fifteen minutes. The mixture is then diluted to fifty millilitres with distilled water, cooled to 25° C. and an aliquot of this solution is tested for fluorescence. A "blank" is run with all determinations. The standard reference solution is the same as for the determinations of the estrogens.

For investigational purposes we used a solution of 25 milligrams of androsterone, 25 milligrams of dehydroandrosterone and 25 milligrams of dehydroandrosterone acetate in one hundred millilitres of absolute ethanol.

The sensitivity with this method is much less than that obtained for estrogens although the reproducibility of results is better. A summary of these results has been given in Table Nineteen, and presented graphically in Graph Two.

Since the greater sensitivity of the estrogens indicated that they were more suitable for assay by fluorescent means, work was discontinued on the androgens and progesterone.

TABLE FIFTEEN

EFFECT OF EVAPORATION IN VACUO ON ANDROSTERONE						
Androsterone micrograms	Evaporation (minutes)	Evaporation °C.	Galvanometer Readings 1		2	
62.5	15	98	16	16	24	27
125.0	15	98	39	38	27	28
187.5	15	98	42	43	60	61
250.0	15	98	63	62	61	74
62.5	30	98	18	18	24	25
125.0	30	98	21	32	28	19
187.5	30	98	47	38	43	39
250.0	30	98	61	53	70	58
62.5	45	98	17	18	16	17
125.0	45	98	30	21	35	14
187.5	45	98	46	55	60	51
250.0	45	98	78	67	66	60
250.0	30	75	25	26	27	24
250.0	45	75	32	32	37	34

TABLE SIXTEEN

EFFECT OF THE REACTION TEMPERATURE AND PERIOD REQUIRED FOR REACTION WITH ANDROSTERONE [N]

Androsterone micrograms	Reaction Temp. °C.	Reaction Time (Minutes)	Galvanometer Readings			
250	100	10	26	26	32	32
250	100	20	25	25	29	28
250	100	30	40	44	47	47
250	120	10	19	31	29	26
250	120	20	38	37	34	35
250	120	30	37	42	48	47
250	75	20	19	24	16	14
250	75	30	32	32	23	26
250	75	45	36	36	32	37
250	Room Temp. (25)	30	44	37	--	--
125	"	30	22	20	20	21
250	Room Temp. (25)	60	42	41	42	42
125	"	60	26	26	25	26

[N] Solution of androsterone evaporated in vacuo for fifteen minutes on a water bath.

[N] Heated with one millilitre of 95% sulfuric acid and diluted to 100 millilitres with distilled water.

TABLE SEVENTEEN

THE EFFECT OF LIGHT AND DARKNESS ON INTENSITY OF FLUORESCENCE N			
Androsterone micrograms	Condition during reaction	Time for reaction (minutes)	Galvanometer Readings
125	In light	immediately	20 20 20 22
250	In light	immediately	32 34 33 32
125	In light	15	15 17 14 14
250	In light	15	32 32 33 32
125	In dark	30	14 14 -- --
250	In dark	30	28 28 -- --
125	In light	40	13 13 -- --
250	In light	40	29 29 -- --
125	In dark	60	15 15 -- --
250	In dark	60	32 31 -- --

N One millilitre of 95% sulfuric acid added to an alcoholic solution of androsterone and the final mixture diluted to 100 millilitres with distilled water.

TABLE EIGHTEEN

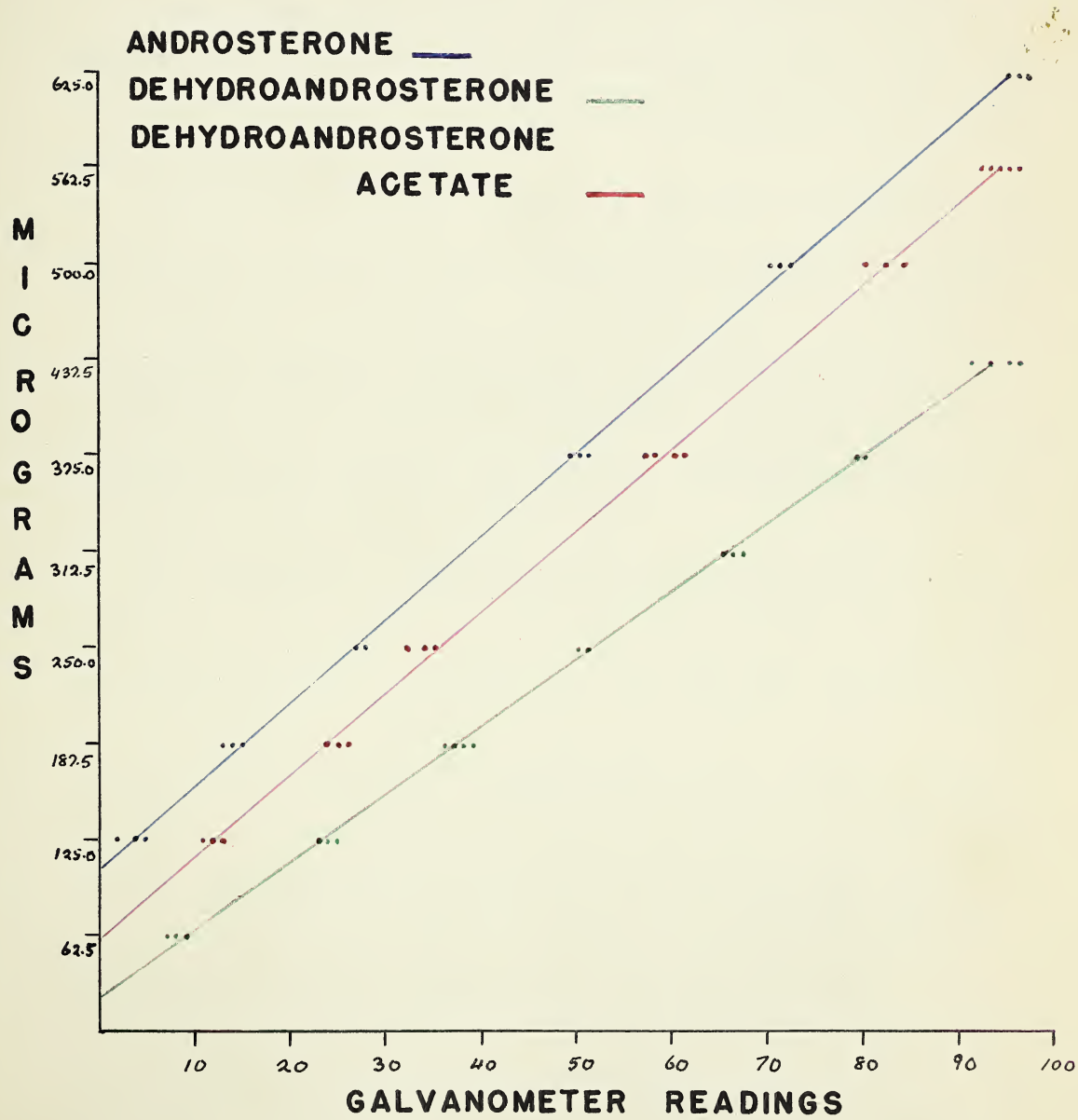
FLUORESCENCE PRODUCED BY ONE MILLILITRE OF 95% SULFURIC ACID N								
Androsterone micrograms	Galvanometer Readings							
162.5	18	16	22	21	19	20	17	18
125.0	29	29	27	30	31	31	31	31
250.0	60	59	50	59	61	60	61	60
312.5	78	78	83	83	82	79	83	82
375.0	83	87	82	84	83	87	86	86

N One millilitre of 95% sulfuric acid added to an alcoholic solution of androsterone which was allowed to stand for fifteen minutes and then diluted to fifty millilitres with distilled water.

TABLE NINETEEN

PHOTOFLUOROMETRIC ASSAY OF CRYSTALLINE ANDROGENS IN ALCOHOL					
Androsterone Micrograms	Galvanometer Readings				
125.0	4.0	2.0	5.0	4.0	5.0
187.5	13.0	13.0	15.0	15.0	14.0
250.0	28.0	27.0	27.0	27.0	27.0
375.0	50.0	51.0	49.0	50.0	50.0
500.0	70.0	70.0	71.0	72.0	72.0
625.0	96.0	96.0	97.0	95.0	95.0
Dehydroandrosterone Micrograms					
62.5	8.0	8.0	8.0	9.0	7.0
125.0	23.0	24.0	23.0	24.0	25.0
187.5	36.0	37.0	38.0	39.0	38.0
250.0	51.0	51.0	50.0	50.0	51.0
312.5	66.0	65.0	66.0	66.0	67.0
375.0	80.0	80.0	79.0	80.0	80.0
437.5	93.0	95.0	91.0	93.0	96.0
Dehydroandrosterone Acetate Micrograms					
125.0	13.0	11.0	11.0	12.0	11.0
187.5	25.0	24.0	26.0	24.0	25.0
250.0	34.0	32.0	32.0	35.0	34.0
375.0	57.0	60.0	58.0	61.0	58.0
500.0	82.0	84.0	82.0	80.0	80.0
562.5	94.0	95.0	92.0	93.0	96.0

GRAPH 2


ANDROSTERONE
DEHYDROANDROSTERONE
DEHYDROANDROSTERONE ACETATE
MICROGRAMS
625.0
562.5
500.0
432.5
375.0
312.5
250.0
187.5
125.0
62.5
10
20
30
40
50
60
70
80
90
100
GALVANOMETER READINGS

SECTION TWO

Jailer (60) in a preliminary paper reported a method for the fluorometric estimation of the estrogenic hormones using 60% sulfuric acid and a heating period of five minutes. The reported sensitivity of this method was greater that the procedure developed in our laboratory and was considered worthy of investigation. Our inital examination of this method was hampered by the scarity of details provided by Jailer (60) but the later appearance of a more detailed account (61) permitted a more thorough examination.

Jailer (61) claimed, in his investigation, to have developed a method whereby he could determine a concentration as low as 0.1 microgram of estrone and estradiol and as low as 0.5 microgram of estriol. The maximum concentrations giving a reading which was within the range of the galvanometer was 0.2 micrograms in the case of the first two estrogens and 2.0 micrograms in the case of the latter.

The intensity of fluorescence was determined in a Coleman Photofluorometer, Model 12 using B-2, PC-9A filter combination. The fluorescence in biological extracts was read by Jailer at two different wave lengths, 436 mu (B-2 filter) and 365 mu (B-1 filter). The non-specific fluorescence of the reagent blanks and other non-estrogenic substances was found to be similiar at the two wave-lengths while that of the estrogens was quite specific at 436 mu. Thus, by reading the solutions with the two filters the blank fluorescence could be deducted.

Attempts to reproduce the results obtained by Jailer with this method were unsuccessful. As shown in Table Twenty it was necessary to employ a concentration of 0.0120 micrograms of

TABLE TWENTY

AN INVESTIGATION OF A PROCEDURE REPORTED BY JAILER (60)						
Estrone micrograms	B-1 Filter		B-2 Filter		Fluorescence due to Estrone	
0.20	100+	100+	25	25	--	--
0.15	100+	100+	23	23	--	--
0.10	100+	100+	24	24	--	--
0.075	100+	100+	20	20	--	--
0.050	100+	100+	18	18	--	--
0.025	100+	100+	18	18	--	--
0.018	100+	100+	17	17	--	--
0.012	82	82	16	16	66	66
0.009	75	75	15	15	60	60
0.006	69	69	14	14	55	55
0.003	62	62	14	14	48	48

TABLE TWENTY-ONE

A COMPARISON OF RESULTS OBTAINED WITH DIFFERENT LAMP INTENSITIES												
Lamp left at full intensity			"STD" knob set at 100 with quinine reference standard				"STD" knob set at at minimum point					
Estrone micrograms	B-1		B-2		B-1		B-2		B-1		B-2	
0.025	100+	100+	18	18	100+	100+	12	12	65	65	10	10
0.010	82	82	16	16	72	72	10	10	48	48	8	8

TABLE TWENTY-TWO

READINGS OBTAINED WITH "STD" KNOB AT A MINIMUM						
Estrone micrograms	B-1 Filter		B-2 Filter		Fluorescence due to Estrone	
0.05	100+	100+	12	12	--	--
0.04	94	94	11	11	83	83
0.03	79	79	10	10	69	69
0.02	65	65	10	10	55	55
0.01	48	48	8	8	40	40

estrone to obtain a reading within the range of our galvanometer.

Plotting the results of these readings graphically showed that a linear relationship did not exist between intensity of fluorescence and concentration of estrone. Extrapolation of the curve to the axis showed that the point of intersection was at a galvanometer reading of approximately 42 which would appear to indicate zero concentration of hormone was still showing a reading of 42.

In his report on this method Jailer made no mention of the use of a standard reference solution. Accordingly, in our attempts to reproduce his results we did not employ such a solution. However, we felt that by employing a standard reference to reduce the lamp intensity, results more nearly approaching those reported by this worker might be obtained. Our first attempt was to adjust the "STD" knob to a galvanometer reading of 100 using a standard reference solution of 0.001 milligrams percent quinine sulfate in 0.1 N sulfuric acid which had been succesfully employed in our initial procedure. To accomplish this we were forced to adjust the "STD" knob using the B-1, PC-1 filter pair. Using the B-2, PC-9A combination the deflection of the galvanometer was so great that no adjustment to 100 could be made. With the B-1, PC-9A combination the reading was extremely low and could not be adjusted satisfactorly. While the results obtained in this instance were somewhat lower than those shown with the same concentrations by the first method the results did not agree with those reported by Jailer (Table Twenty).

Our next attempt to reproduce Jailer's results was to reduce the lamp intensity to its minimum point. This was

accomplished by rotating the "STD" knob as far as possible without shutting off the Photofluorometer. This procedure enabled us to obtain readings over a concentration range of 0.01 to 0.04 micrograms of estrone which is one-fiftieth the concentration examined by Jailer (Table Twenty-two).

In this instance also we were unable to plot intensity against concentration in such a manner that the curve obtained would split the axis at zero. With this curve, extrapolation showed a reading of 26 with zero concentration of estrone.

We have, therefore, been unable to reproduce the results obtained by Jailer.

Technique Two

The high sensitivity of Jailer's method indicated the possibility that our original procedure (Technique One) might be improved.

Further investigation of our procedure showed that results within galvanometer range were obtainable using one-tenth the original concentration if the mixture was diluted to ten millilitres instead of the one hundred millilitres previously employed. The effect of the amount of acid was also further tested; and an examination of the time required to complete the reaction was made.

The filter combination was the same as in our initial investigations and the same strength standard reference solution was used. Attempts to use other filter combinations, such as that used by Jailer, failed to give satisfactory results. The B-1, PC-9A pair gave readings with the "blank" far beyond the range of the galvanometer and made adjustment to a reading of zero impossible. The readings obtained with other filter combinations gave lower values than those obtained

with the B-1, PC-1 combination.

TABLE TWENTY-THREE

TIME REQUIRED TO COMPLETE FLUORESCENT DEVELOPMENT Ⅱ			
Estrone Micrograms	Temp. of Heating °C.	Heating Period (mins.)	Galvanometer Readings
2.5	120	5	12 14 14 14
2.5	120	10	13 13 12 13
2.5	120	20	14 13 13 13
2.5	120	20	16 14 14 14

Ⅱ Estrone solution evaporated to dryness in vacuo, heated with one millilitre of 95% sulfuric acid and diluted to ten millitres.

An examination of the time required for reaction showed that maximum results could be achieved with a five minute heating period (Table Twenty-three). A longer period of heating at 120°C. did not increase the intensity of fluorescence above that obtained with the five minute period.

TABLE TWENTY-FOUR

RELATIVE FLUORESCENT INTENSITIES WITH DIFFERENT VOLUMES OF SULFURIC ACID Ⅱ					
Estrone Micrograms	Millilitres of 95% sulfuric acid	Galvanometer Readings			
2.5	0.5	9	9	9	9
2.5	1.0	14	14	14	14
2.5	1.5	16	16	16	16
2.5	2.0	18	19	18	19
2.5	3.0	20	20	20	20
2.5	4.0	23	28	22	20

Ⅱ Estrone solution evaporated to dryness in vacuo, heated for five minutes at 120° C. with the acid and diluted to ten millilitres.

Investigations of the volume of acid required to develop the greatest intensity of fluorescence showed a 50% increase

in intensity resulting when three millilitres of acid were employed in place of the one millilitre previously utilized (Table Twenty-Four). Four millilitres of acid increased the intensity but decreased the reproducibility. Three millilitres of acid was thus selected as a suitable volume to be added to the reaction mixture.

TABLE TWENTY-FIVE

RELATIVE INTENSITIES WITH DIFFERENT STRENGTHS OF ACID N			
Estrone Micrograms	Strength of Acid % v/v.	Millilitres of acid	Galvanometer Readings
2.5	50	3	5 7 6 5
2.5	60	3	14 13 14 14
2.5	80	3	17 17 17 16
2.5	95	3	20 20 20 20

N Estrone solution evaporated to dryness in vacuo, heated for five minutes at 120° C. with the acid and diluted to ten millilitres.

Weaker concentrations of sulfuric acid produced readings of lower intensity, but satisfactory agreement of results was obtained regardless of the strength of acid employed (Table Twenty-Five).

TABLE TWENTY-SIX

TEMPERATURE OF HEATING REQUIRED TO DEVELOPE MAXIMUM FLUORESCENCE N			
Estrone Micrograms	Temperature of Heating °C.	Heating Period (mins.)	Galvanometer Readings
2.5	room temp.	5	3 5 7 0
2.5	98	5	12 11 13 13
2.5	120	5	20 20 20 20
2.5	150	5	15 13 10 17

N Estrone solution evaporated to dryness in vacuo, heated with three millilitres of 95% sulfuric acid and diluted to ten millilitres.

In view of the smaller concentrations of estrogens involved in these assays the question arose as to the necessity of still employing a reaction temperature of 120°C. As shown by Table Twenty-six, however, better reproducibility and greater intensity were obtained at this temperature than at higher or lower temperatures. The reproducibility of results at 98°C. was still quite satisfactory but the intensity of fluorescence was approximately forty percent less than that resulting when a temperature of 120°C. was employed.

TABLE TWENTY-SEVEN

THE INHIBITORY EFFECT OF WATER ON THE DEVELOPMENT OF FLUORESCENCE ✠				
Procedure	Galvanometer Readings			
Evaporation in vacuo before heating	20	19	20	20
Non evaporation of solvent before heating	3	5	2	6
Dilution to ten millilitres before heating	8	10	7	8

✠ Three millilitres of sulfuric acid used and the mixture heated at 120°C. for five minutes and diluted to ten millilitres.

Finklestein et al (62) have reported that water has an inhibitory effect on the production of fluorescence. An investigation showed that the presence of water in the mixture during the period of reaction decreased sensitivity and reproducibility (Table Twenty-seven). We continued, therefore, to evaporate an aliquot of the estrogen solution to dryness in vacuo before adding the acid.

From the results obtained in these investigations the following procedure was developed which was considerably more

sensitive than Technique One.

Technique Two

An aliquot of a one milligram percent solution of the estrogen in ethanol is evaporated to dryness in vacuo on a boiling water bath. Three millilitres of concentrated sulfuric acid are added and the mixture is heated with agitation for five minutes at 120°C. The tube is removed and cooled immediately under cold water. Five millilitres of distilled water are added and the mixture is again cooled under the cold water tap to 25°C.

A "blank" is run with all determinations and a standard reference solution of 0.001 milligrams percent quinine sulfate in 0.1 N sulfuric acid is used to adjust the lamp intensity. The "STD" knob is adjusted to give a galvanometer reading of 100 with this solution.

The addition of the distilled water to the sulfuric acid must be performed cautiously to avoid splattering. We found it satisfactory to add the water from a burrette along the side of the tube so as to form a layer of water on top of the acid. The solution was then mixed carefully with a fine glass stirring rod.

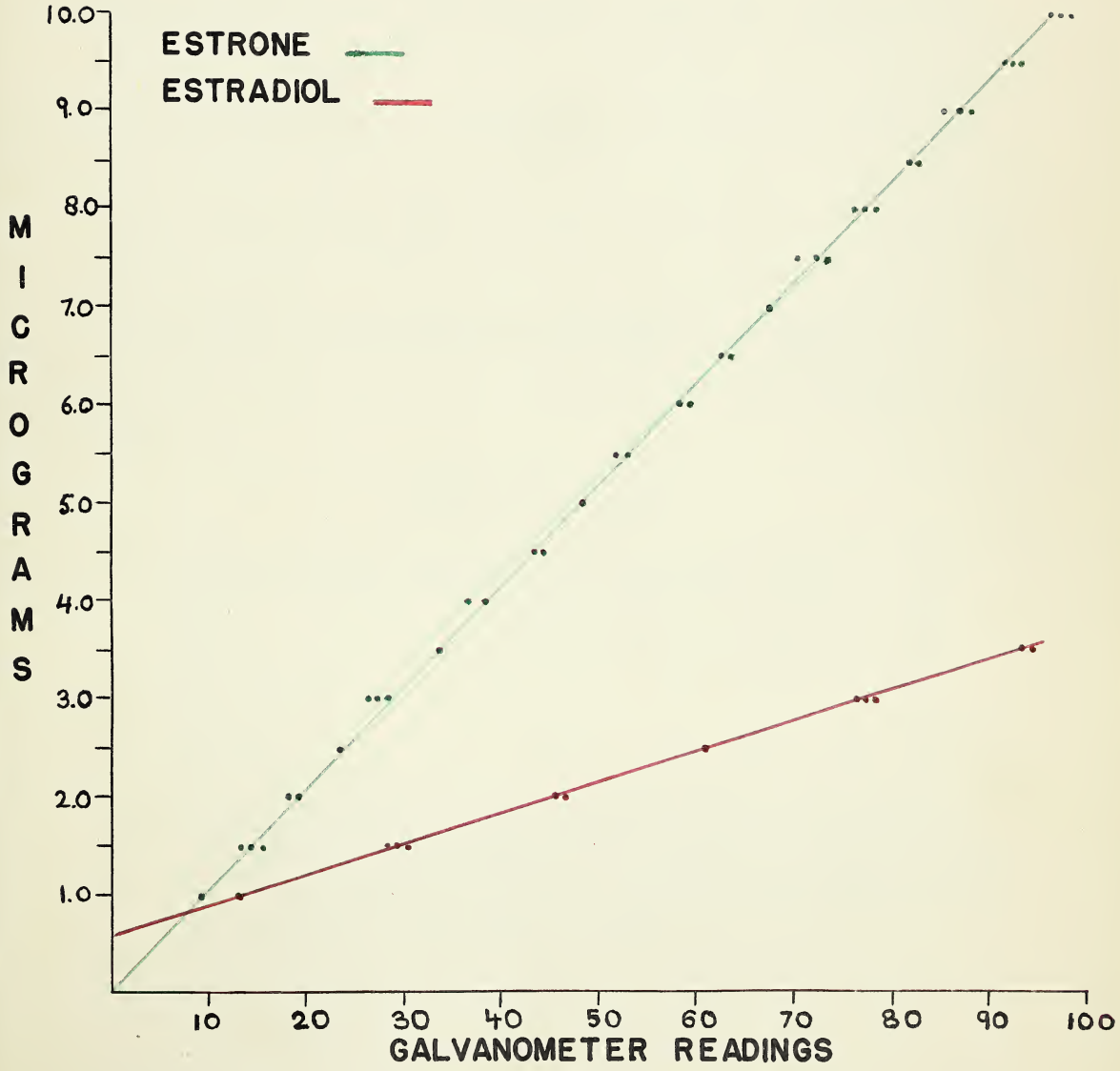
Table Twenty-eight presents the results obtained with estrone and estradiol using Technique Two. It can be noted from these tables that this method is ten times more sensitive for estrone and two times more sensitive for estradiol than Technique One. In Technique One, we were forced to employ concentrations of from 10 to 65 micrograms of estrone and 10 to 70 micrograms of estradiol to cover the complete galvanometer scale. In Technique Two, 1 to 10 micrograms of estrone and 1 to 3.5 micrograms of estradiol are all that are necessary.

TABLE TWENTY-EIGHT

CALIBRATION OF THE PHOTOFLUOROMETER FOR ESTROGENS BY TECHNIQUE TWO

Estrone Micrograms	Galvanometer Readings				
1.0	9.0	9.0	9.0	9.0	9.0
1.5	14.0	14.0	15.0	14.0	13.0
2.0	19.0	18.0	19.0	19.0	19.0
2.5	23.0	23.0	23.0	23.0	23.0
3.0	28.0	28.0	28.0	27.0	26.0
3.5	33.0	33.0	33.0	33.0	33.0
4.0	38.0	38.0	36.0	38.0	38.0
4.5	43.0	44.0	43.0	43.0	44.0
5.0	48.0	48.0	48.0	48.0	48.0
5.5	53.0	54.0	53.0	54.0	54.0
6.0	58.0	59.0	58.0	58.0	58.0
6.5	63.0	63.0	63.0	62.0	63.0
7.0	67.0	67.0	67.0	67.0	67.0
7.5	72.0	72.0	70.0	73.0	72.0
8.0	77.0	78.0	76.0	77.0	77.0
8.5	82.0	82.0	81.0	82.0	82.0
9.0	87.0	85.0	88.0	87.0	88.0
9.5	92.0	93.0	91.0	92.0	92.0
10.0	98.0	97.0	97.0	97.0	96.0
Estradiol Micrograms	**Galvanometer Readings**				
1.0	13.0	13.0	13.0	13.0	13.0
1.5	29.0	29.0	30.0	29.0	28.0
2.0	45.0	46.0	45.0	45.0	45.0
2.5	61.0	61.0	61.0	61.0	61.0
3.0	77.0	78.0	78.0	77.0	76.0
3.5	93.0	93.0	94.0	93.0	93.0

GRAPH 3


ESTRONE
ESTRADIOL
MICROGRAMS
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
10
20
30
40
50
60
70
80
90
100
GALVANOMETER READINGS

Graph Three shows the results when intensity is plotted against concentration for these two estrogens. It will be noted that estrone obeys Beer's Law; but with estradiol the point of intersection is such that a concentration of 0.6 micrograms would not exhibit fluorescence.

Phosphoric Acid

Finklestein et al (62) reported the use of phosphoric acid to produce fluorescence of the estrogens. For their procedure a Lumetron colorimeter fitted with attachments to convert the apparatus to a photofluorometer had been employed. Two millilitres of the phosphoric acid solution were used. Since the Coleman Photofluorometer requires an eight millilitre aliquot for final analysis we were unable to duplicate this procedure exactly. However, a comparison of the relative intensities of fluorescence developed by phosphoric and sulfuric acids showed the latter to be superior (Table Twenty-Nine).

TABLE TWENTY-NINE

COMPARISON OF SULFURIC AND PHOSPHORIC ACID M				
Acid	Galvanometer Readings			
Phosphoric	5	5	5	5
Sulfuric	21	20	20	20

M 0.25 millilitres of an estrone solution containing 2.5 micrograms evaporated to dryness in vacuo, heated for five minutes at 120°C. with 3 millilitres of acid and diluted to ten millilitres.

SECTION THREE

Assay of Urinary Extracts

In the Kober reaction (46) and its modifications (17, 18) difficulty has been encountered in assaying urinary extracts of estrogens due to the presence of interfering pigments. The pink colour produced by the phenolsulfonic acid reagent on reaction with the estrogens is obscured by brownish pigments present in pregnacy urine. The resulting effect is an overestimation of the estrogen content. Various modifications (17, 19, 47) of this test have been proposed to overcome the effect of this brown colour, either by removal of the brown pigmentation or by attempts to correct for it. Bachman and Petit (21) and Talbot (47) have expressed doubt as to the rationale of using correction equations.

Bachman and Petit (21) have stated that assay procedures based on the Kober reaction are inapplicable to non-pregnant urines. For non-pregnant urines Friedgood and Garst (58) have used ultra-violet absorption while Jailer (60, 61) has applied fluorometric techniques.

It was hoped that some of the disadvantages of other chemical tests could be overcome by the application of our fluorometric procedures to the assay of urinary extracts. Since the important factor in estrogenic tests is the biological potency, it is necessary to correlate all chemical tests with biological activity. For this reason urine from the same normal male was extracted and assayed biologically as well as by fluorescent means.

Assays were performed on twenty-four hour samples of male urine. No attempt was made to estimate separately the amounts

of the various estrogens present in the extract. Extractions were performed so as to maintain the total estrogen content of the extract while removing the androgens and other contaminants by various means. In the procedures where a separation of the estrogen into specific entities arose as a part of the technique, the separate portions were combined and assayed together. The results obtained were expressed in terms of estrone.

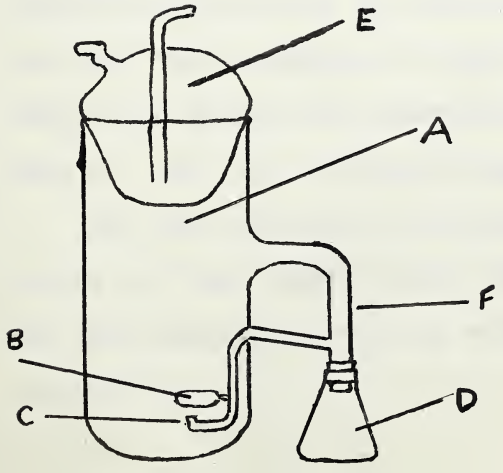
Collection of Samples

No preservative was employed and the samples were kept under refrigeration until the extraction could be performed.

Extraction of the Urine

Our initial extraction procedure was that employed by Leiboff and Tamis (24). This method had previously been found satisfactory in this laboratory for the extraction of estrogens (66). The procedure is a continuous one employing chloroform as the extraction medium.

Preliminary hydrolysis of the urine is performed by adding fifty millilitres of concentrated hydrochloric acid to a five hundred millilitre aliquot of twenty-four urine and evaporating the mixture to one-third its original volume by boiling.



In the extraction apparatus illustrated chloroform is placed in chamber A to a level two inches above plate B. B is a glass plate protecting the opening of the overflow tube C. This

overflow tube, C, leads to an Erlenmeyer flask, D, which serves as the collection vessel.

The hydrolyzed urine is poured into A and forms a layer above the chloroform already present. More chloroform is then slowly added to A until chloroform is seen to pass through tube C into flask D. Enough chloroform is then added to give a volume of about twenty-five millilitres in flask D.

The condenser E is then set in place and both the extraction chamber, A, and collection vessel, D, are gently heated, causing the chloroform to boil gently but steadily.

When the chloroform in A boils it rises partly up into the urine and drops back again. When the temperature of the urine is elevated to that of the boiling chloroform, the chloroform will bubble vigorously through the urine, vaporize, and be liquified by the condenser.

While this is proceeding the chloroform in flask D is vigorously heated and evaporates through the arm F and is liquified by the condenser. The increased volume causes the chloroform to rise in tube C and spill over into the flask.

Leiboff and Tamis (24) have reported that a two hour extraction period is sufficient to extract all the chloroform soluble material and to concentrate it in flask D. We modified the procedure in that we extracted for a three hour period and added the chloroform fraction from flask A to that from D. This was to insure complete extraction(66).

For the purposes of biological assay five millilitres of sesame oil were added to the chloroform extract. The solution was then heated in vacuo on a boiling water bath to remove the chloroform.

Biological Assay

Although a number of biological methods have been described (33-41), the rat vaginal smear method of Allen and Doisy (1), is highly satisfactory for the assay of estrogens and was employed by us throughout.

Young female rats were spayed and smears taken one week later. The rats were not used for assay until all were definitely negative.

We used the following criteria for the classification of vaginal smears (66).

I. Preponderence of leucocytes associated with a few compact and squamous cells.

II. Predominance of compact cells with a few squamous cells and leucocytes.

II+ Compact cells only in large numbers of a characteristic appearance with the stained part drawn in, leaving a white ring around the cell or part of it.

III. Predominance of squamous cells with a few leucocytes and compact cells.

IV. Squamous cells only.

I and II are interpreted as negative.

II+, III, and IV are interpreted as positive.

The smears were taken on the second, third and fourth days after injection. A small medicine dropper was employed for this purpose. A small volume of distilled water was injected gently into the vagina, the contents agitated, and removed. The contents of the dropper were spread on a slide, stained with methylene blue, and examined microscopically.

Five rats were employed for each assay. The material was

administered in one injection subcutaneously under the loose skin of the flank. As an initial dose, 0.2 millilitres of the 5 millilitres of extract were given. When all the rats were negative with this dose, 0.4 millilitres was administered as a second dose. A period of at least ten days was allowed to elapse between the administration of the two doses. The rats were smeared before the administration of the second dose to determine if all were negative.

A total of four extractions of twenty-four male urines were assayed by the Allen and Doisy method (1) and in no instance were we able to obtain a positive response with either a 0.2 or a 0.4 millilitre dose, (Table Thirty), indicating a low estrogenic titre.

TABLE THIRTY

ALLEN-DOISY ASSAY OF EXTRACTS OF TWENTY-FOUR MALE URINE

Extract No.	Rat No.	0.2 millilitre 24	48	72	0.4 millilitre 24	48	72
1	1	1	1	1	1	11	1
6	6	1	1	1	1	1	1
	8	1	1	1	1	1	1
	9	1	1	1	1	1	1
	10	1	1	1	1	1	1
2	12	1	1	1	1	1	1
	15	1	1	1	1	1	1
	17	1	1	1	1	1	1
	18	1	1	11	1	1	1
	19	1	1	1	1	1	1
3	2	1	1	1	11	11	1
	3	11	11	11	1	11	11
	4	1	1	1	1	1	1
	5	11	11	11	11	11	11
	7	1	1	1	1	11	11
4	11	1	1	1	1	1	1
	13	1	1	1	1	1	1
	14	1	1	1	1	1	1
	16	1	1	1	1	1	1
	20	1	1	1	1	1	1

To ascertain the ability of the spayed rats to respond to estrogens, 10 micrograms of estrone were administered to each of the rats used for assay purposes. The estrone was contained in 0.2 millilitre of sesame oil and in every case we obtained positive smears (Table Thirty-one).

TABLE THIRTY-ONE

ALLEN-DOISY ASSAY OF ESTRONE

Rat No.	Classifications		
	24 hours	48 hours	72 hours
1	II+	IV	IV
2	I	IV	IV
3	II	III	IV
4	I	IV	IV
5	II	IV	III
6	II	IV	IV
7	II	IV	IV
8	II+	IV	IV
9	II	IV	IV
10	III	IV	IV
11	II	III	IV
12	I	III	IV
13	II+	IV	IV
14	II	III	IV
15	II	IV	IV
16	II	IV	III
17	II+	IV	IV
18	I	II+	IV
19	II+	IV	IV
20	II	IV	IV

Fluorometric Analysis of Extracts

A series of twenty-four hour male urines extracted by the method of Leiboff and Tamis (24) were assayed by fluorometric

Technique One. For this purpose the chloroform was removed by distillation on a water-bath and the residue was dissolved in 25 millilitres of ethanol. A 0.25 millilitre aliquot of this solution was used for assay.

All results obtained showed no agreement with the similiar series run using the Allen and Doisy (1) assay technique. In every instance the determined concentrations were several thousand times the normal twenty-four hour value reported in the literature (67).

TABLE THIRTY-TWO

ASSAY OF ESTROGENIC EXTRACTS BY FLUOROMETRIC TECHNIQUE ONE

Extract Number	Galvanometer Readings					Ave. Conc. as Estrone
1	63	65	68	65	69	4.48 mgms.
2	35	37	32	32	35	2.34 mgms.
3	73	76	72	72	71	4.92 mgms.
4	75	74	74	74	73	5.00 mgms.

Following the development of fluorometric Technique Two, additional attempts were made to assay estrogenic extracts of twenty-four hour male urine. The same extraction was utilized in our initial attempts to obtain a satisfactory extract for fluorometric assay without any better success.

Further attempts at purification and decolorization of the final extract did not improve the findings. While the results were somewaht lower the concentrations, as determined, were still many times the known daily excretory value of estrone in male urine.

The purification procedures involved treatment of the final extract with charcoal and a chromatographic treatment (27) of the residue. These purifications were carried out separately but in several instances a combination of the two procedures were used. That is, the extract was passed through the chromatographic column and the eluate decolorized with charcoal.

In Table Thirty-Three are compiled the results obtained with several different extraction and purification procedures. Extracts 1 and 2 show the results with the Leiboff and Tamis (24) method without any further purification. Numbers 3 and 4 are extracts obtained by the method of Leiboff and Tamis but modified by treatment with charcoal (Extract 3); and by passing the solution through the chromatographic column of Stimmel (27),(Extract 4). Extract 5 was prepared by chromatographic purification and decolorization with charcoal.

Departing somewhat from the usually accepted methods of extraction of the estrogens from urine by parition between immiscible solvents, Freed et al (22) have proposed a method involving the precipitation of the estrogens with a fifty percent sodium tungstate solution. No preliminary hydrolysis of the urine was performed by these workers. We introduced this modification to ensure complete extraction of the estrogenic hormones (16). The technique employed consisted of refluxing the urine for ten minutes with 15 volumes percent hydrochloric acid and cooling the reaction mixture under the cold water tap immediately (20). The acidified urine was then treated with 5 N sodium hydroxide solution so as to

have the urine at approximate neutrality.

The combined ether extracts were evaporated to dryness and the residue was dissolved in twenty-millilitres of ethanol. A 0.25 millilitre aliquot of this solution was tested for fluorescence initially and where the reading obtained was beyond galvanometer range, smaller volumes of the solution were analyzed.

The readings obtained with this extraction procedure were also much higher than could be attributed to estrogens (Extract 6). Treating an extract obtained in this manner with charcoal until the extract was colorless still gave very high readings (Extract 7).

As there was a possibility that androgens were extracted along with the estrogens and were contributing in part to the fluorescence, an attempt was made to remove these by shaking the final ether extract with four separate portions of 2 N sodium hydroxide solution. The alkaline solution was then acidified with concentrated hydrochloric acid and extracted twice with twenty-five millilitre volumes of ethyl ether. The ether extracts were combined and washed twice with five millilitre volumes of 5% sodium carbonate solution and twice with five millilitre volumes of water. The washings were discarded. The residue was then evaporated to dryness and dissolved in twenty-five millilitres of ethanol for fluorometric assay. The results obtained with 0.25 millilitre aliquots of this solution are shown in Table Thirty-three (Extract 8).

A number of extraction methods described in the literature (19-21 , 68,69) were next investigated in the hope of finding a method where the final results would compare

favourably with results determined by biological assay.

In all these methods the extracts are obtained in the usual manner involving preliminary hydrolysis of the urine followed by extraction with water-immiscible organic solvents. The procedures differ, however, in the purification techniques employed to get final extracts free of contaminants.

Venning et al (19) and Stimmel (27) favour the use of butyl alcohol for the extraction of the estrogenic hormones from urine. This solvent is reported to extract fewer chromogenic pigments than other fat solvents. Further purification (19) is accomplished by washing the extract with sodium hydroxide solutions to effect the separation of the estrogens from the androgens. The phenolic nature of ring A of the estrogenic hormones enables them to react with solutions of the alkaline hydroxides to form the water soluble sodium salts. The androgens do not react and are left in the organic solvent.

The final extract was, in our opinion, no less pigmented than an extract obtained by other methods (24). Results obtained again (Extract 9) greatly overestimated the normal estrone content of male urine and treatment of an extract with charcoal (Extract 10) did not significantly reduce the readings.

Stimmel (27) has applied chromatographic treatment to effect the purification of the estrogenic extracts. The ether extract is dissolved in benzene and passed through a column, 200 centimetres in length containing Alumina (Brockman) which has been previously moistened with twenty millilitres of benzene. The column is then fixed with a further fifty

millilitres of benzene. The estrogenic hormones are left behind on the column and their subsquent removal is effected by means of various strength solutions of absolute methanol in benzene. The three estrogens, estrone, estradiol and estriol may be collected separately by using different mixtures of methanol and benzene. As we desired to examine the total estrogenic content of urine we used a mixture consisting of thirty parts of methanol and seventy parts benzene. This mixture has been reported to remove quantitatively all the estrogenic hormones.

Extract 11 in Table Thirty-three shows the results with the Stimmel (27) procedure. The eluate was a dark brown colour and this pigmentation perhaps contributed to the high figures. Decolorization of the extract with charcoal (Extract 12) produced somewhat lower figures but the agreement with bioassay was still not satisfactory.

Cohen and Marrian (17) recommend the acidification of the alkaline solution of the estrogens and washing of this solution with toluene. This washing is reported to remove such substances as pregnanediol and cholesterol which inhibit colour development in the Kober (46) reaction.

No better success was achieved with this procedure(Extract 13) or with subsequent treatment of the final extract with charcoal for decolorization purposes (Extract 14, Table Thirty-three).

Bachman and Petit (21) have taken advantage of the weakly acidic properties of the estrogens and their relatively low solubility in mineral acids to effect purification. The benzene extract of urine is washed with 10% v/v of sulfuric

acid which removes much pigmented and chromogenic material from this solution. Estriol is previously removed by washing with 9% sodium carbonate as this estrogen is more soluble in the mineral acids and might be lost.

Similiar results were obtained with this procedure as with the previous extractions (Extract 15). Treatment of the final extract with charcoal reduced the reading somewhat (Extract 16). Passing a solution of the extract through a chromatographic column also caused a reduction (Extract 17). Treatment of the eluate from Extract 17 with charcoal made little improvement (Extract 18).

Beall and Edson (68) found that saturation of the toluene extract with sodium chloride prevented the removal of estrone and estradiol on subsequent washing with N/10 sodium hydroxide. At the same time much undesirable acidic material was removed. However, we obtained high estimates of estrone concentration using this procedure (Extract 19) and on subsequent treatment with charcoal (Extract 20).

Mather (69) employed petroleum ether for purification purposes. This solvent is used to remove lipids and phenolic steroids but did not have the effect of reducing the titre significantly (Extract 21). Treatment of the extract with charcoal did not produce the desired results (Extract 22).

None of the extraction and purification procedures examined gave products whose assay by our fluorometric procedure could be accepted as correct. In every instance the readings obtained greatly overestimated the reported estrone excretory value for normal male's urine (67). It would seem that much non-estrogenic material remains in these extracts

TABLE THIRTY-THREE

THE PHOTOFLUOROMETRIC ASSAY OF NORMAL MALE URINARY EXTRACTS BY TECHNIQUE TWO

Extract Number	Urinary Volume (mls.)	Conc. as Estrone (mgms)
1	760	1.2312
2	800	1.3600
3	610	0.5490
4	670	0.5628
5	670	0.2144
6	660	1.8000
7	660	1.0500
8	710	0.6600
9	630	1.6500
10	630	1.3000
11	700	0.4000
12	760	0.3100
13	560	1.6000
14	685	1.9000
15	855	0.1780
16	735	0.0800
17	590	0.0900
18	600	0.0840
19	670	1.8250
20	670	1.5250
21	610	1.4500
22	640	2.2250

and contributes to the fluorescence when treated with sulfuric acid.

At this time Bates and Cohen (63) published a procedure for the fluorometric estimation of estrogens and in order for us to evaluate this report the work on urinary extracts was discontinued.

SECTION FOUR

Technique Three

The publication by Bates and Cohen (63) of a method for the fluorometric estimation of the estrogens indicated that a simpler procedure than that developed by us was possible.

The procedure as described by these workers is as follows: An aliquot of an alcoholic solution of the estrogens is placed in the cuvette and to this is added one millilitre of 95% sulfuric acid. The contents of the tube are mixed and heated in a water bath at 80° C. for ten minutes. The tube is then removed, cooled, and eight millilitres of 65% sulfuric acid are added. The tube is again cooled and analyzed to determine the intensity of fluorescence.

These workers reported a linear relationship between intensity and concentration for both estrone and estradiol over a range of one to ten micrograms.

The filter utilized was the same B-1, PC-1 combination with which we had obtained the best success.

The 65% sulfuric acid was made up in the ratio of 65 parts of 95% sulfuric acid to 35 parts of water as recommended. While the resulting concentration is not exactly 65% w/w or v/v, we will refer to the strength of the sulfuric acid by this figure hereafter.

To test this procedure we heated a series of aliquots of one milligram percent solutions of estrone in 95% ethanol at 80° C. for ten minutes. The concentrations tested ranged from one to ten micrograms and were contained in 0.1 to 1.0 millilitres of 95% ethanol. The sensitivity of our apparatus was such that the maximum concentration giving a reading

TABLE THIRTY-FOUR

INTENSITY OF FLUORESCENCE AT 80°C. WITH TECHNIQUE THREE N					
Estrone Micrograms	Galvanometer Readings				
0.5	26.0	20.0	22.0	19.0	20.0
1.0	39.0	38.0	42.0	39.0	38.0
1.5	59.0	50.0	58.0	59.0	59.0
2.0	78.0	79.0	78.0	77.0	78.0
2.5	100.0	98.0	99.0	98.0	98.0

N To an aliquot of estrone solution in alcohol add one millilitre of 95% sulfuric acid. Heat the mixture for five minutes at 80°C. in a water bath and add eight millilitres of 65% sulfuric acid. Cool.

TABLE THIRTY-FIVE

INTENSITY OF FLUORESCENCE AT 98°C. WITH TECHNIQUE THREE N					
Estrone Micrograms	Galvanometer Readings				
0.5	30.0	30.0	30.0	29.0	30.0
1.0	61.0	62.0	61.0	61.0	62.0
1.5	86.0	88.0	86.0	86.0	85.0
2.0	100	100	100	100	100

N To an aliquot of estrone solution in alcohol add one millilitre of 95% sulfuric acid. Heat the mixture for five minutes in a boiling water bath and add eight millilitres of 65% sulfuric acid. Cool.

within galvanometer range was 2.5 micrograms (Table Thirty-four).

It seemed possible that the different readings obtained by different workers, Jailer, Bates and Cohen and ourselves were due to differences in apparatus. To test this possibility we compared results obtained with our apparatus to those obtained with a similiar apparatus in the Department of Biochemistry using the standard reference solution. Quite different readings were obtained. It would seem that it is necessary to calibrate each apparatus, and that repeatedly, in order to detect failure.

As shown also in Table Thirty-four there was poor agreement in readings given by the same concentration of estrone. Investigation showed the non-reproducibility was overcome by raising the temperature of the reaction mixture to that of a boiling water bath (98°C.) from 80°C. (Table Thirty-five). Not only was the reproducibility much better but sensitivity was increased.

No mention was made by Bates and Cohen (63) of the use of a standard reference solution. In our attempts to reproduce the results obtained by these workers we made use of the quinine sulfate solution previously employed as a reference solution. Adjustment of the lamp intensity, and thus instrument sensitivity, by means of such a solution produces less variation in readings.

In our previous techniques (One and Two) we had set the galvanometer to a reading of 100 with the quinine reference standard. With Technique Three it was found more suitable to set the galvanometer at 80 or approximately the minimum point

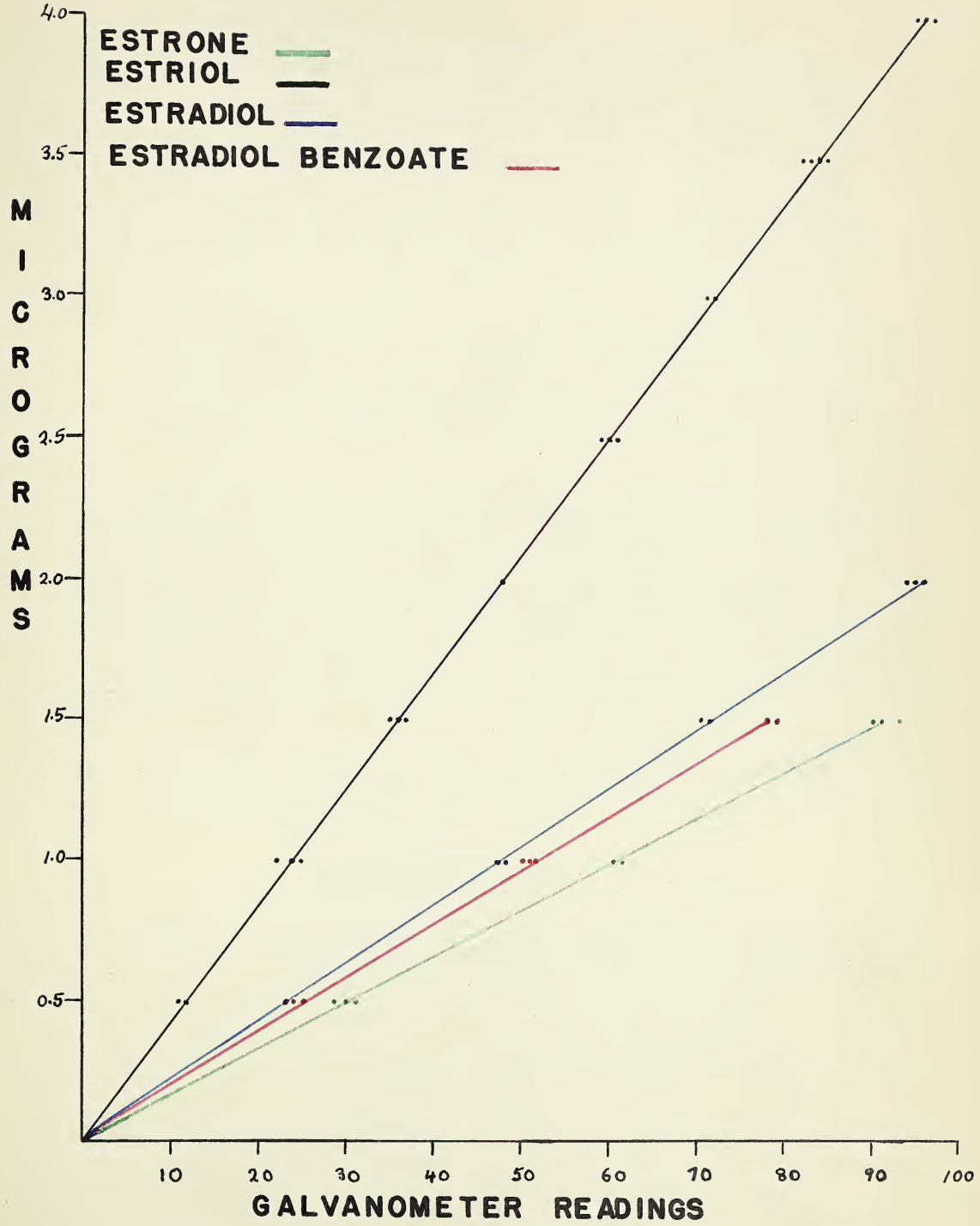
TABLE THIRTY-SIX

CALIBRATION FC THE PHOTOFLUOROMETER FOR ESTROGENS BY TECHNIQUE THREE Ⅱ

Estrone Micrograms	Galvanometer Readings					
0.5	30.0	30.0	30.0	30.0	29.0	31.0
1.0	61.0	62.0	61.0	61.0	62.0	61.0
1.5	91.0	93.0	91.0	91.0	90.0	91.0
2.0	100+	100+	100+	100+	----	----
Estradiol Micrograms	**Galvanometer Readings**					
0.5	24.0	24.0	24.0	24.0	24.0	24.0
1.0	48.0	49.0	49.0	48.0	48.0	48.0
1.5	71.0	72.0	72.0	72.0	72.0	72.0
2.0	96.0	94.0	96.0	96.0	94.0	95.0
2.5	100+	100+	100+	100+		
Estriol Micrograms	**Galvanometer Readings**					
0.5	12.0	11.0	11.0	12.0	12.0	12.0
1.0	24.0	24.0	22.0	24.0	24.0	25.0
1.5	36.0	37.0	36.0	36.0	35.0	36.0
2.0	48.0	48.0	48.0	48.0	48.0	48.0
2.5	60.0	60.0	61.0	59.0	60.0	60.0
3.0	72.0	71.0	72.0	72.0	72.0	71.0
3.5	84.0	82.0	84.0	84.0	83.0	85.0
4.0	96.0	95.0	97.0	96.0	96.0	97.0
Estradiol Benzoate Micrograms	**Galvanometer Readings**					
0.5	26.0	26.0	26.0	27.0	26.0	26.0
1.0	52.0	51.0	51.0	52.0	52.0	53.0
1.5	78.0	78.0	78.0	79.0	79.0	78.0
2.0	100+	100+	100+	100+	----	----

Ⅱ To an aliquot of estrogen solution in alcohol add one millilitre of 95% sulfuric acid. Heat the mixture for five minutes in a boiling water bath and add eight millilitres of 65% sulfuric acid. Cool.

GRAPH 4


ESTRONE
ESTRIOL
ESTRADIOL
ESTRADIOL BENZOATE
MICROGRAMS
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
10
20
30
40
50
60
70
80
90
100
GALVANOMETER READINGS

to which the "STD" knob may be rotated. This setting gave results more nearly approximating those of Bates and Cohen than did a setting of 100.

This method of assay was found applicable to estrone, estradiol, estriol and estradiol benzoate. In every instance the reproducibility was very good; and the figures obtained obey Beer's Law. The results obtained in the calibration of the apparatus for these hormones are presented in Table Thirty-six and Graph Four.

The ability of Bates and Cohen to obtain a satisfactory sensitivity without preliminary evaporation of the aliquot of estrogen solution to dryness raised the question as to the results obtainable if this procedure were used. It was also desired to know whether the volume of alcohol employed would have any effect on the final intensity. In Table Thirty-seven is presented data showing the effects of evaporation as well as the results obtained when the estrone was present in volumes of alcohol ranging from 0.1 to 0.5 millilitres. Estrone was used as the test material and appropriate "blanks" were run in each case.

TABLE THIRTY-SEVEN

M

THE EFFECT OF ALCOHOL VOLUME ON THE INTENSITY OF FLUORESCENCE				
Millilitres of alcohol	Galvanometer Readings			
0.10	61.0	61.0	61.0	61.0
0.25	61.0	61.0	61.0	61.0
0.50	60.0	60.0	60.0	60.0
Evaporation	100+	100+	100+	100+

M To one microgram of estrone is added One millilitre of 95% sulfuric acid. Heat mixture for five minutes in a boiling

water bath and add eight millilitres of 65% sulfuric acid. Cool.

Evaporation of the alcoholic solution of estrone to dryness before the addition of the 95% sulfuric acid was found to increase greatly the intensity of fluorescence. Readings in every instance were beyond galvanometer range. The readings obtained with the estrone in solution were considerably lower, however, than when the evaporation process was used. The amount of ethanol, 0.1 to 0.5 millilitres, used produced no significant difference in the readings obtained.

Despite the higher intensity achieved by the evaporation method we did not change to this technique. The simplicity of the method, when the hormone is in solution, we felt outweighed the greater sensitivity obtained by evaporation. Also, we considered the assay procedure, as performed, to be of sufficient sensitivity for most purposes.

The initial mixing of the one millilitre of 95% sulfuric acid and the eight millilitres of 65% sulfuric acid produces cloudiness throughout the reaction mixture. This cloudiness contributes considerably to the recorded intensity and gives an inaccurate estimation of the true concentration of hormone present. The cloudiness takes several minutes to disappear and final readings should not be recorded until all cloudiness has disappeared.

SECTION FIVE

The Assay of Commercial Preparations of Estrogens

The potency of commercial preparations of estrogens is expressed both in terms of milligrams and International Units of estrone or of estradiol benzoate, depending on the material present. In the case of extracts of conjugates which have been obtained from pregnant mare's urine and other sources, the potency is expressed in terms of equivalents of International Units of estrone. Present methods of assay of these products usually employ the Allen and Doisy (1) vaginal smear technique. Several chemical procedures (70, 71) for the determination of estrogenic preparations have been described in the literature and these and other modifications, the details of which are not available outside their laboratories, are employed by various manufacturers.

Types of preparations at present manufactured for clinical purposes include.

1. Estradiol tablets.
2. Estradiol benzoate or estradiol dipropionate in solution in various oils.
3. Estradiol ointments.
4. Estrone in solution in various oils.
5. Estrone in suspension in aqueous mediums.
6. Estriol capsules.
7. Sodium estrone sulfate tablets.
8. Conjugated estrogens in solution in various oils.
9. Conjugated estrogens in tablet form.

The strength of these preparations range from 0.1 milligrams (1000 I.U.) to 5 milligrams (50,000 I.U.).

The extreme sensitivity manifested by our method of assay suggested the possibility of its adaptation to the assay of these products. Inasmuch as we were able to determine a concentration as low as 0.5 micrograms the method appeared to possess the necessary qualifications for the estimation of the strength of individual tablets or ampoules without the necessity of combining several of these in order to have sufficient for assay purposes.

Estradiol Tablets

Control samples were made up consisting of estradiol in an intimate mixture with a vehicle composed of equal parts of corn starch and lactose. This mixture would appear to approximate the tablets on the market as they are in the form of tablet triturates. No attempt was made to mould these control mixtures into tablet form, the extraction being made from the powder.

The strength of commercial tablets marketed are 0.1 milligram, 0.2 milligram, and 0.5 milligrams and the individual tablets weigh approximately 80 milligrams. The procedure employed for the preparation of the control mixture was to weigh one milligram of estradiol and add it to a mixture of equal parts of corn starch and lactose. To prepare a mixture containing 0.5 milligrams of estradiol, the hormone would be mixed with 159.0 milligrams of the vehicle and this mixture would then be divided into two parts of equal weight, each containing 0.5 milligrams of estradiol.

Organic solvents would appear to be suitable for extraction purposes since estrogens are soluble in these materials, whereas the inert vehicle is not. The solvents investigated

were chloroform, anaesthetic ether and 95% ethanol.

The mixture was suspended in twenty-five millilitres of the solvent and agitated intermittently for one half hour. The mixture was then filtered through a micro sintered glass filter using negative pressure. The residue remaining in the Erlenmeyer was washed several times with ten millilitre lots of the solvent. The washings were added to the main portion of the solvent and evaporated to a volume of two or three millilitres with the aid of gentle heat. The remainder of the solvent was removed by means of a stream of compressed air over the surface of the liquid. The purpose of this last step was to avoid charring and possible destruction of the estradiol. The residue was dissolved in sufficient ethanol to make a one milligram percent solution. 0.1 millilitre aliquots of this solution were then assayed fluorometrically using Technique Three.

Examination showed anaesthetic ether to be the most satisfactory extraction medium (Table Thirty-nine). Ethanol 95% gave results varying from 25% to 50% in excess of the theoretical concentration. Addition of the 95% sulfuric acid to an aliquot of the extract made with this solvent showed evidence of charring.

Chloroform, on the other hand, produced readings lower than the theoretical concentration. However, agreement of assays run in duplicate was good and there was no evidence of charring. Whether the low readings were due to incomplete extraction or to the presence of inhibitory substances in the chloroform is unknown.

Anaesthetic ether gave results showing recovery of 94-

101% of the known concentration of estradiol. As this appeared to be within the limits of experimental error, ether was considered satisfactory and used throughout.

Examination showed that there was no necessity of allowing the mixture to stand one-half hour before filtration. Shaking the mixture vigorously with twenty-five millilitres of anaesthetic ether in a fifty millilitre Erlenmeyer completely extracted the estradiol. The Erlenmeyer was closed with a rubber stopper or cork covered with tin foil.

To test the possibility that part of the fluorescence was contributed by the ether or vehicle, several extracts were made of "blank" samples of the vehicle. No fluorescence was exhibited by these "blanks".

An examination of a number of commercial tablets extracted by the above technique proved successful. The only change necessary in the technique was breaking up the tablet into a fine powder before extraction. This was accomplished by means of a fine glass stirring rod.

The results of these assays are compiled in Table Forty. In several instances we extracted more than one tablet at a time to test the applicability of the technique to tablets of greater concentration than we had available. Satisfactory extractions resulted in every instance.

Estrone Aqueous Suspensions

Estrone aqueous suspension are marketed by several firms and consist of a suspension of pure crystalline estrone in isotonic sodium chloride solution. In one preparation of this type, 5.25% gum acacia is present to decrease the

TABLE THIRTY-EIGHT

SELECTION OF THE SOLVENT FOR EXTRACTION OF ESTRADIOL FROM TABLETS					
Solvent	Known Concentrations (mgms.)	Determined Concentrations % of Known			
		Series One		Series Two	
Chloroform	0.500	83.0	84.8	83.0	83.0
	0.500	78.8	78.8	78.8	78.8
Ethanol	0.500	150.0	124.2	111.0	122.2
Anaesthetic ether	0.500	97.2	100.0	100.0	100.0
	0.500	100.0	100.0	100.0	100.0
	0.500	100.0	97.2	100.0	100.0
	0.500	95.3	93.2	95.3	93.2
	0.500	97.2	97.2	97.2	101.8
	0.500	100.0	100.0	100.0	100.0
	0.500	101.8	100.0	100.0	100.0
	0.500	100.0	100.0	100.0	100.0
	0.500	101.8	101.8	101.8	101.8
	0.500	100.0	100.0	100.0	97.2
	0.200	100.0	100.0	100.0	100.0
	0.200	100.0	101.8	101.8	100.0
	0.200	97.2	101.8	100.0	100.0
	0.200	100.0	100.0	100.0	101.8
	0.200	97.2	97.2	97.2	97.2

TABLE THIRTY-NINE

PHOTOFLUOROMETRIC ASSAY OF COMMERCIAL ESTRADIOL TABLETS BY TECHNIQUE THREE

Company	Stated Concentrations (mgms.)	Galvanometer Readings						Determined Concentrations as % of the Known
Ciba	0.5	48	47	48	48	47	49	99.4
Ciba	0.5	48	49	49	48	48	50	101.3
Ciba	0.5	48	48	49	48	48	48	100.3
Ciba	0.5	48	48	48	48	48	48	100.0
Ciba	0.5	49	47	47	47	47	48	98.4
Ciba	0.5	48	49	48	48	48	48	100.3
Ciba	0.5	48	48	48	47	49	48	99.8
Ciba	0.5	48	48	48	48	48	49	100.3
# Ciba	1.0	48	47	49	48	48	47	99.3
Ciba	1.0	48	47	49	48	48	48	99.8
## Ciba	1.5	48	48	48	48	47	47	99.6
Ciba	1.0	48	48	47	48	48	48	99.5
### Ciba	2.0	47	47	47	48	48	48	98.6
Ciba	0.2	48	48	48	48	48	48	100.0
Ciba	0.2	48	48	48	48	48	48	100.0
Ciba	0.2	48	49	48	48	48	48	100.3
Ciba	0.2	48	48	48	47	49	48	99.8
Ciba	0.2	49	49	50	49	49	49	102.1
Ayerst	0.1	49	48	48	49	48	48	100.6
Ayerst	0.1	48	48	48	48	48	48	100.0
Ayerst	0.1	48	48	48	49	47	47	99.4
Ayerst	0.1	48	48	48	48	48	48	100.0
Ayerst	0.1	48	48	48	48	47	48	99.5

\# Two tablets extracted
Three tablets extracted
Four tablets extracted.

tendency of the crystals to settle out of suspension.

Mather (69) in his investigation on the partition of estrone between immiscible solvents had found estrone to be quantitatively removed from an aqueous medium with any one of a number of organic solvents including ether. The success we had achieved with anaesthetic ether in the extraction of estradiol from powders and tablets lead us to employ this solvent for estrone.

The strength of the control preparations was two milligrams per millilitre of vehicle. Two types of vehicles for this class of preparation were investigated. A simple vehicle of 0.9% sodium chloride solution and a similiar solution containing 5.25% gum acacia. To provide a more suitable working condition, the one millilitre volume of vehicle was diluted to ten millilitres with distilled water.

To determine the amount of ether required for complete extraction several different methods were employed.

1. Extraction with three separate volumes of anaesthetic ether of ten millilitres each.
2. Extraction with three separate volumes of twenty-five, fifteen and ten millilitres respectively.
3. Extraction with two separate volumes of twenty-five millilitres each.

The separate portions of ether were combined and evaporated nearly to dryness at a low temperature. The remaining ether was then removed by passing a stream of compressed air over the surface of the residue. The residue was dissolved in twenty-five millilitres of ethanol and suitable

dilutions were made so as to give a final solution containing ten micrograms of estrone per millilitre. Aliquots of this final solution were assayed by Technique Three.

Extraction of a suspension of estrone in simple sodium chloride solution by the first method produced results averaging approximately 80% of the known concentration (Table Forty). The results did not agree well in duplicate and this made us suspect the presence of some inhibitory substance in the final extract. Backwashing of the ether with ten millilitres of distilled water in two separate five millilitre. portions was found to overcome this variance and the determined concentrations agreed well with the known concentrations (Table Forty).

Extraction with the thirty millilitre volume of anaesthetic ether of a suspension of estrone in gum acacia did not effect complete extraction of the hormone (Table Forty). Increasing the number of washings with distilled water did not remove any material which might be inhibiting the fluorescent development and did not produce the desired readings. Extraction with a twenty-five millilitre volume followed by a fifteen and a ten millilitre volume of anaesthetic ether was found to effect complete extraction. The three portions of anaesthetic ether were combined and backwashed with two lots of five millilitres each of distilled water. The same results could be obtained with two extractions, utilizing twenty-five millilitres for each extraction (Table Forty).

A total of five ampoules were assayed in the Photofluorometer by Technique Three to determine the estrone

TABLE FORTY

EXTRACTION OF CONTROL PREPARATIONS OF ESTRONE IN AQUEOUS SUSPENSION

Procedure	Vehicle	Known Concentration (mgms.)	Determined Concentrations % of the Known			
			Series One		Series Two	
Extraction with 3 x 10 ml. lots of ether	Sodium Chloride Solution	2.000	78.8	89.0	84.8	70.5
	"	2.000	91.6	80.8	84.8	76.6
As above but was with 10 mls. of water	"	2.000	100.0	97.2	101.8	97.2
	"	2.000	97.2	100.0	100.0	100.0
	"	2.000	100.0	100.0	100.0	100.0
	"	2.000	97.2	97.2	100.0	97.2
	"	2.000	100.0	100.0	101.8	100.0
	Acacia Solution	2.000	70.5	72.3	68.0	70.5
	"	2.000	76.6	76.6	76.6	76.6
Extraction with 1 x 25, 1 x 15 and 1 x 10 ml. lots of ether	"	2,000	93.5	95.2	93.5	93.5
	"	2.000	100.0	100.0	100.0	101.8
Extraction with 2 x 25 ml. lots of ether	"	2.000	100.0	97.2	97.2	100.0
	"	2.000	101.8	101.8	101.8	101.8
	"	2.000	100.0	100.0	100.0	101.8

content. The vehicle used for suspending purposes in these was sodium chloride solution containing 5.25% gum acacia. The extractions were performed with two separate volumes of twenty-five millilitres of anaesthetic ether which were combined and backwashed with ten millilitres of distilled water in two separate five millilitre volumes. The readings range from 96.6 to 102.9% of the labelled strength (Table Forty-One).

The alcoholic solution of the final extract was diluted to give a solution containing ten micrograms per millilitre; and 0.1 millilitre aliquots of this solution were assayed by Technique Three. If the extraction were 100% such an aliquot should give a reading of 61. This is the reading obtained with a similiar solution of crystalline estrone in ethanol (Table Thirty-eight).

TABLE FORTY-ONE

PHOTOFLUOROMETRIC ASSAY OF COMMERCIAL SUSPENSIONS OF ✠ ESTRONE IN AQUEOUS MEDIA BY TECHNIQUE THREE

Galvanometer Readings						Average Determined Concentration % of the Known
60	62	61	61	61	60	99.8
63	63	63	62	62	63	102.9
61	61	61	61	61	61	100.0
60	61	61	61	60	61	99.6
59	59	58	59	59	59	96.6

✠ Abbott's Estrone Aqueous Suspensions. 2 milligrams of estrone in isotonic saline solution containing 5.25% gum acacia.

Estrone in Oil

A number of ampoules of crystalline estrone in solution in either peanut or sesame oil were made available to us for assay purposes. The several strengths provided were 1, 0.2 and 0.1 milligrams per millilitre of oil.

An attempt was made to extract the estrone from these solutions following a procedure outlined by Carol and Rotondaro (71). The technique employed was as follows:-

One millilitre of the oil solution of estrone was measured into a 125 millilitre separatory funnel containing twenty-five millilitres of petroleum ether (boiling point 35-60° C.). Ten millilitres of 2 N sodium hydroxide solution were added, the mixture was shaken vigorously for two minutes, and the two layers were allowed to separate completely. The aqueous layer was transferred to a second 125 millilitre separatory funnel. The extraction was repeated with two additional ten millilitre portions of 2 N sodium hydroxide solution, each of which was added to the second funnel. The petroleum ether solution was discarded. A 50% v/v solution of sulfuric acid was added to the alkaline solutions until a permanent opalesence formed (acid to litmus). The mixture was cooled thoroughly and twenty-five millilitres of anaesthetic ether were added. The mixture was shaken throughly for one minute and the two layers were allowed to separate. The acid layer was transferred to a second 125 millilitre separatory funnel and the extraction was repeated with another twenty-five millilitres of anaesthetic ether. The acid layer was discarded. The ether layer was washed with two, five millilitre portions

of 5% sodium carbonate solution and then with two five millilitre portions of distilled water. The ether solution was transferred to a 50 millilitre beaker and evaporated to a volume of three or four millilitres. The final few millilitres of liquid were removed by directing a stream of compressed air over the surface.

Carol and Rotondaro (71) were able to report recoveries of estrone ranging from 98% to 101% of the known concentrations in control samples prepared in sesame oil. The assay procedure employed was of the gravimetric type and good agreement was obtained in duplicate samples. The method was effective for concentrations of from one to ten milligrams of estrone.

Assay of solutions of estrone containing 1 milligram per millilitre in either peanut or sesame oil indicated a degree of extraction (Table Forty-two) comparable to that obtained by Carol and Rotondaro (71). The control samples were prepared by dissolving the crystalline estrone in anaesthetic ether, adding the required amount of oil and removing the ether by gently heating the mixture on a hot-plate. One millilitre portions of the resulting solution in oil were extracted and assayed by Technique Three.

Commercial ampoules containing one milligram of estrone per millilitre in both peanut and sesame oils were extracted by the above method and assayed by Technique Three. The residue remaining after removal of the ether was dissolved in sufficient alcohol to make a one milligram percent solution; and 0.1 millilitre aliquots of this solution were

assayed in the Photofluorometer. The assay results, reported as a percentage of the label statement are compiled in Table Forty-two.

Although Carol and Rotondaro (71) had not employed this procedure for the gavimetric assay of smaller quantities than one milligram of estrone we attempted to assay 0.2 milligram per millilitre solutions in both peanut and sesame oils. Initial attempts produced results higher than the known concentration. In some instances the discrepancy was as much as 60% greater and the results also did not agree well in duplicate (Table Forty-three).

Vegetable oils are known to produce fluorescence and it was thought that either the oily vehicle itself or fatty acids present in the final extract might be contributing to the fluorescent intensity. We repeated these assays with a second oil but with the same anomalous results (Table Forty-three).

Several approaches appeared possible in our examination of the probable causes of the variance.

1. The fluorescence due to the oil could be corrected for by using a solution made from a "blank" oil.
2. Extraction with fewer volumes of 2 N sodium hydroxide solution.
3. Extraction with N instead of 2 N sodium hydroxide solution.
4. Extraction with fewer and/or smaller volumes of anaesthetic ether.
5. Washing with more volumes and/or stronger strengths of sodium carbonate solution.

TABLE FORTY-TWO

PHOTOFLUOROMETRIC ASSAY OF SOLUTIONS OF ESTRONE IN OIL

Company	Vehicle	Known Conc.	Galvanometer Readings ※						Average of Determined Conc. % of the Known
Control	Sesame	1.000	64	64	64	64			105.4
Control	Sesame	1.000	61	61	61	61			99.6
Control	Sesame	1.000	61	61	61	61			100.0
Control	Sesame	1.000	62	61	61	61			100.5
Control	Sesame	1.000	61	61	62	62			100.9
Control	Peanut	1.000	61	61	61	61			100.0
Control	Peanut	1.000	60	60	61	61			99.1
Control	Peanut	1.000	62	61	61	61			100.5
Control	Peanut	1.000	61	62	62	62			101.4
Control	Peanut	1.000	62	60	61	61			100.0
Abbotts	Sesame	1.0	56	56	56	56	56	56	92.0
Abbotts	Sesame	1.0	61	61	61	63	61	61	100.9
Abbotts	Sesame	1.0	63	63	63	63	63	61	103.1
Abbotts	Sesame	1.0	62	60	61	61	61	61	100.0
Abbotts	Peanut	1.0	61	61	61	61	61	61	100.0
Abbotts	Peanut	1.0	63	60	61	61	61	61	100.3
Abbotts	Peanut	1.0	60	60	59	60	61	59	98.1
Abbotts	Peanut	1.0	61	61	61	61	61	61	100.0
Parke Davis	Peanut	1.0	60	60	60	61	61	61	99.1
Parke Davis	Peanut	1.0	60	60	60	60	60	59	98.8
Parke Davis	Peanut	1.0	61	61	61	61	61	58	99.2
Parke Davis	Peanut	1.0	61	61	61	61	61	61	100.0
Parke Davis	Peanut	1.0	59	59	61	59	58	58	97.1

※ 0.1 millilitre aliquot of a 1 milligram percent solution assayed which should give a reading of 61 if the extraction was 100%.

The "blank" extract was dissolved in twenty millilitres of ethanol corresponding to the amount of alcohol present in a one milligram percent solution of estrone prepared using 0.2 milligram of crystalline estrone. The intensity of fluorescence produced by "blank" portions of both peanut and sesame oils showed no agreement between duplicate aliquots of the same extract or between different extracts. Since it was impossible to record the same value for the "blank" oil in each assay we were unable to use this technique.

The Carol and Rotondaro (71) procedure had employed three separate extractions of the petroleum ether solutions with ten millilitre portions of 2 N sodium hydroxide solution. On the assumption that fewer fatty acids might be extracted with one or two extractions instead of the three previously used we prepared extracts using this technique. The results obtained with two extractions were still much higher than the theoretical or known concentrations and the variance between duplicate determinations was still marked. When one extraction with 2 N sodium hydroxide was used the recoveries were less than 100% and the extreme variance between duplicates made us fear that most of the fluorescence was due to fatty acids or oil (Table Forty-Three).

Extraction with one, twenty-five millilitre portion of anaesthetic ether or with three, fifteen millilitre volumes did not overcome the variance. The final extract was dissolved in twenty millilitres of ethanol and assayed fluorometrically by Technique Three with no better sucess (Table Forty-Three).

Increasing the number of washings with sodium carbonate solution to four washings with five millilitre volumes of 5% sodium carbonate solution did not decrease the variance between duplicates or reduce the intensity of fluorescence to a reading proportional to the estrone present. The use of three volumes of five millilitres each of 10% sodium carbonate solution was not satisfactory because an emulsion formed and it was difficult to get complete separation of the two layers (Table Forty-three).

Visual examination of the results remaining after evaporation of all these extractions showed a slight oily film to be present in nearly every case. This film had also been present in the residue remaining after ether extraction of the controls containing one milligram of estrone. However, the results obtained with these extracts on fluorescent analysis of an alcoholic solution of this residue had shown no variance between duplicates and good agreement with known concentrations. The solution of the residue for these previous assays had been made in one hundred millilitres of ethanol, whereas, for the assay of 0.2 milligram per millilitre solutions of estrone, the residue had been dissolved in twenty millilitres of ethanol. The purpose of this was to have the resulting solution contain ten micrograms of estrone per millilitre of ethanol which would have the same strength as the solution used for the calibration of the Photofluorometer with estrone. By using such a solution, 0.1 millilitre aliquots should give a reading of 61 on the galvanometer, or the same as the solution of crystalline estrone in ethanol. This procedure

TABLE FORTY-THREE

INVESTIGATION OF VARIABLES EFFECTING THE FLUORESCENCE DISPLAYED BY EXTRACTS OF 0.2 MILLIGRAM PER MILLILITRE SOLUTIONS OF ESTRONE IN OIL

Procedure	Peanut Oil Sample	Determined Concentrations as % of the Known			
		Series One		Series Two	
#Carol and Rotondaro (71)	1	142.8	162.5	112.0	140.0
# "	1	150.0	128.0	134.8	123.5
# "	2	158.0	141.8	151.0	131.8
#Extract with 2 x 10 millilitre lots of 2 N NaOH	2	115.0	123.8	141.5	108.4
#Extract with 1 x 10 millilitre lots of 2 N NaOH	2	54.0	77.2	88.8	61.0
#Extract with 1 x 25 millilitre lots of anaesthetic ether	2	101.8	123.8	133.0	108.4
#Extract with 3 x 15 millilitre lots of anaesthetic ether	2	105.4	114.8	116.2	110.0
#Washing with 4 x 5 millilitre lots of 5% sodium carbonate	2	110.0	130.0	119.0	103.8
#Washing with 3 x 5 millilitre lots of 10% sodium carbonate	2	113.4	116.2	105.4	123.8
##Carol and Rotondaro (71) but run against "blank" oil	2	120.0	120.0	120.0	118.0
##Carol and Rotondaro (71) but run against "blank" oil	2	120.0	121.5	120.0	120.0
###Carol and Rotondaro (71)	2	101.8	101.8	100.0	100.0

#Residue dissolved in twenty millilitres of ethanol and a 0.1 millilitre aliquot assayed by Technique Three.
##Residue dissolved in fifty millilitres of ethanol and a 0.25 millilitre aliquot assayed by Technique Three.
###Residue dissolved in one hundred millilitres of ethanol and a 0.50 millilitre aliquot assayed by Technique Three.

TABLE FORTY-FOUR

INVESTIGATION OF VARIABLES EFFECTING THE FLUORESCENCE DISPLAYED BY EXTRACTS OF 0.2 MILLIGRAM PER MILLILITRE SOLUTIONS OF ESTRONE IN OIL N

1. 95% ethanol assayed by Technique Three.
2. An aliquot of an alcoholic solution of the extract obtained from the "blank" oil assayed by Technique Three. The volume of this aliquot was the same as the volume of alcohol used in 1.
3. Duplicate of 2 assayed at the same time.

Oil	Millilitres added to Residue	Extract One			Galvanometer Readings Extract Two			Extract Three		
		1	2	3	1	2	3	1	2	3
Sesame	20	38	53	64	38	81	73	38	60	72
Sesame	50	41	53	53	41	53	53	41	53	53
Sesame	100	44	44	44	44	44	44	44	44	44
Peanut	50	41	53	53	41	53	53	41	53	53
Peanut	100	44	44	44	44	43	44	44	44	44

N The aliquot of alcoholic solution used for assay is the amount that would contain one microgram of estrone if this were present.

simplified comparisons.

Investigation of the effect of dissolving the residue in various volumes of ethanol showed that agreement in duplicate could be achieved by dissolving the residue in fifty millilitres instead of twenty millilitres as previously. 0.25 millilitre aliquots of this fifty millilitre solution, however, showed readings of 73 instead of 61 or a determined concentration of 120% of the known strength. Employing a "blank" prepared from either peanut or sesame oils this discrepancy was reduced to nil in the majority of

TABLE FORTY-FIVE

ASSAY OF CONTROL AND COMMERCIAL PREPARATIONS OF ESTRONE IN OIL BY TECHNIQUE THREE

Company	Oil	Known Conc. (mgms.)	Galvanometer Readings						Average Determined Conc. % of the Known
Control	Sesame	0.200	61	61	60	59			98.9
Control	Sesame	0.200	61	61	60	60			99.2
Control	Sesame	0.200	61	63	61	62			101.4
Control	Peanut	0.200	62	62	62	63			102.3
Control	Peanut	0.200	61	61	62	61			100.4
Control	Peanut	0.200	61	59	61	61			99.3
Abbotts	Peanut	0.2	61	61	61	61	61	61	100.0
Abbotts	Peanut	0.2	61	61	61	60	61	61	99.6
Abbotts	Peanut	0.2	58	58	59	58	58	58	95.7
Abbotts	Peanut	0.2	61	60	62	61	61	61	100.0
Abbotts	Peanut	0.2	61	61	61	61	61	59	99.5
Control	Sesame	0.100	28	29	30	31	31	30	98.3
Control	Sesame	0.100	29	29	29	31	31	31	98.0
Control	Sesame	0.100	30	30	30	30	32	30	100.9
Control	Sesame	0.100	31	30	30	30	31	31	101.0
Control	Sesame	0.100	30	30	28	30	29	28	96.7
Control	Peanut	0.100	30	30	30	30	30	30	100.0
Control	Peanut	0.100	30	30	33	30	30	30	101.5
Control	Peanut	0.100	30	39	31	30	30	31	100.0
Abbotts	Sesame	0.1	31	29	30	30	30	30	100.0
Abbotts	Sesame	0.1	30	30	31	31	32	30	101.6
Abbotts	Sesame	0.1	32	30	30	30	30	29	100.3
Abbotts	Sesame	0.1	30	30	31	31	31	31	101.3
Abbotts	Sesame	0.1	31	31	32	31	29	29	100.6

[M] Residue dissolved in 100 millilitres of ethanol and 0.5 millilitre aliquots assayed by Technique Three.

cases, and the concentrations determined were approximately 100% of the known concentration (Table Forty-three).

Examination of the "blank" oils showed that solutions of the residue in fifty millilitres of ethanol gave readings of 12 in every case but solution in one hundred millilitres reduced the fluorescence displayed by these to zero. Solution of the residue in twenty millilitres of ethanol produced readings varying greatly in every assay (Table Forty-four). We, therfore, abandoned the previous procedure of making one milligram percent solutions of estrone and dissolved the residue in one hundred millilitres of ethanol. The same procedure was also used for extracts of 0.1 milligram per millilitre solutions of estrone in oil with satisfactory results (Table Forty-five).

A number of commercial ampoules of estrone in both peanut and sesame oils were examined by the above procedure. The strength of these preparations was 0.1 or 0.2 milligrams per millilitre of crystalline estrone. The results of these assays together with the results of assay of controls are presented in Table Forty-Five. All determined concentrations agreed satisfactorily with the labelled strength.

Estradiol Monobenzoate

Since estradiol monobenzoate is insoluble in solutions of alkali hydroxides it cannot be extracted from oils in a similiar manner to estrone. Therefore, a method for its estimation in oil solution would require one or the other of the following two methods:-

1. An assay based on the difference in fluorescence exhibited by the oil solution and the oil itself.

2. Hydrolysis of the estradiol monobenzoate to estradiol and separation from the products of hydrolysis of the oil.

Since the molecular weight of estradiol monobenzoate is 376.77 and that of estradiol is 272.2, 1.38 milligrams of the monobenzoate yields 1 milligram of estradiol. Control samples were prepared containing 1 milligram of estradiol monobenzoate per millilitre of peanut oil. This amount of estradiol monobenzoate would be equivalent to 0.723 milligrams of estradiol.

To examine the first method one millilitre of peanut oil was diluted to one hundred millilitres with ethanol and 0.1 millilitre aliquots of this solution were tested for fluorescent intensity by Technique Three. The readings obtained were all beyond galvanometer range and the meter could not be adjusted to zero with the "STD" knob. Thus the possibility of employing a method of assay based on difference in fluorescence displayed by the oil solution and the oil itself was not possible.

Hydrolysis

Elvidge (70) had described a procedure for the hydrolysis of estradiol monobenzoate to estradiol and extraction of the salt from the resultant soap. He obtained readings 42% in excess of the theoretical concentration and attributed the error to the presence of fatty acids in the final extract. These fatty acids gave absorption, on spectrophotometric analysis, in the same region as the estradiol and thus the error arose.

An attempt to obtain an extract by the method of Elvidge (70) produced an oily residue on evaporation of the

final ether solution. Assay of an 0.1 millilitre aliquot of this residue dissolved in one hundred millilitres of ethanol produced readings beyond the range of the galvanometer. This would indicate a concentration of at least 100% in excess of the known concentration.

Our next attempts were aimed at removing the fatty acids formed during the saponification process. The saponification was performed by refluxing the oil for thirty minutes with twenty millilitres of N/2 alcoholic potassium hydroxide on a boiling water bath (70).

A solution of lead acetate on addition to a solution of fatty acids will form lead soaps, which are insoluble in both water and organic solvents. The United States Pharmacopoeia XIII in its monograph for peanut oil describes a process for precipitating the fatty acids of this oil by the addition of a hot solution of lead acetate. The soap formed by saponification is dissolved in fifty millilitres of hot water, and hydrochloric acid is added until the fatty acids separate as an oily layer. These fatty acids are dissolved in ether, and a hot alcoholic solution of lead acetate is added. The mixture is then allowed to stand for twelve hours and the insoluble lead soap filtered off.

An effort was made to adapt the United States Pharmacopoeia procedure for the precipitation of fatty acids to the removal of these fatty acids from our extract of estradiol monobenzoate in oil solutions.

Extract One

The soap obtained by the saponification of the oils was dissolved in fifty millilitres of hot water and hydrochloric acid was added until the fatty acids just precipitated. The solution was extracted with three, twenty-five millilitre portions of anaesthetic ether and backwashed with two, five millilitre portions of distilled water. The washings were discarded. To the ether solution was added two grammes of lead acetate in twenty-five millilitres of hot ethanol. The mixture was agitated and allowed to stand for twelve hours. The precipitate which formed was filtered off and the ether evaporated at a low temperature. The residue was dissolved in one hundred millilitres of ethanol and 0.1 millilitre aliquots of this solution were assayed by Technique Three.

The readings obtained agreed well in duplicate but the determined concentration was 35% in excess of the known concentration of estradiol monobenzoate in the control sample (Table Forty-six).

Two other attempts to obtain a satisfactory extract in this manner produced results 69% and 38% in excess of the known concentration (Table Forty-six).

Extract Two

In our second attempt to get a pure extract the initial extraction was performed as above. The final residue remaining after the evaporation of the ether, however, was dissolved in twenty-five millilitres of petroleum ether and a further extraction was performed by the method of Carol and Rotondaro

(71) for the removal of estrone from oil solutions.

Determined concentrations with two attempts were 37.6 and 40.8% of the theoretical concentration of estradiol monobenzoate.

Extract Three

The addition of a solution of lead acetate to the fatty acids immediately after acidification of the aqueous solution of soap, without preliminary extraction with ether, produced a dense precipitate of lead chloride. The amount of this precipitate could be reduced by controlling the amount of acid added prior to the addition of the lead acetate solution. Adding the acid drop by drop until the fatty acids just precipitated was found to produce the least amount of precipitate.

The solution and precipitate were extracted with three, twenty-five millilitre volumes of anaesthetic ether. The ether extracts were combined, filtered through a small plug of absorbent cotton and backwashed with ten millilitres of distilled water. The ether was then evaporated to dryness and the residue dissolved in one hundred millilitres of ethanol. 0.1 millilitre aliquots of this solution were assayed by Technique Three.

The results obtained did not agree well in duplicate and averaged 54% in excess of the known concentration (Table Forty-six).

Two other attempts failed to produce a satisfactory extract. In these instances evaporation of the ether left an oily solution indicating that the fatty acids had not been precipitated.

TABLE FORTY-SIX

ASSAY OF SOLUTIONS OF ESTRADIOL BENZOATE IN OIL CONTAINING ONE MILLIGRAM PER MILLILITRE OF OIL N

Method of Extraction	Galvanometer Readings				Average of Determined Concentrations % of the Known
Elvidge (70)	100	100	100	100	----------
1	48	48	49	48	135.0
1	59	59	58	59	169.0
1	49	46	49	47	138.0
2	12	14	11	11	37.6
2	12	14	11	11	40.8
3	56	51	53	60	154.0
NN 4	31	31	31	31	88.9
N N 4	29	30	29	29	83.7
4	48	48	48	47	137.6
4	53	53	53	53	150.7
5	22	26	19	23	65.0
5	40	34	38	36	106.8

N Residue dissolved in one hundred millilitres of ethanol and 0.1 millilitre aliquots assayed by Technique Three.

N N Results run in two series on the same extract.

Extract Four

The cloudy nature of the final ether solution and the slight solubility of lead acetate in ether indicated that perhaps this material was contributing to the fluorescence. Backwashing of this ether solution with five millilitre volumes of distilled water until the solution was clear produced a determined concentration of 88.9% and 83.7% of the known in duplicate determinations. However, we were unable to repeat these results and other determinations averaged 37.6 and 50.7% in excess of the known concentration (Table Forty-six).

Extract Five

The insolubility of lead carbonate in ether led to an attempt to precipitate any lead acetate that might be present by the addition of 10% sodium carbonate solution. The ether solution was washed with five millilitre volumes of 10% sodium carbonate solution until no more precipitate formed. The ether solution was then filtered through absorbent cotton and backwashed with two, five millilitre portions of distilled water. Several results were obtained ranging from 65% of the known concentration to 6.8% in excess with poor agreement in duplicate.

No further investigation of this latter step was undertaken as in many instances the addition of sodium carbonate solution produced a permanent emulsion. This would appear to indicate that the fatty acids were not completely precipitated and were reacting with the sodium carbonate solution to form a soap.

Efforts to develope an extraction procedure for estradiol

monobenzoate have not been successful.

Preparations of Conjugated Estrogens

Certain commercial preparations used for estrogenic activity consist of conjugated estrogens and complex estrogens as found in pregnant mare's urine. These preparations are stated by the manufacturers to contain mostly estrone with varying amounts of equilin, equilenin and potassium estrone sulfate. The activity of these preparations is expressed in terms of International Units of estrone and the potency is determined by biological assay.

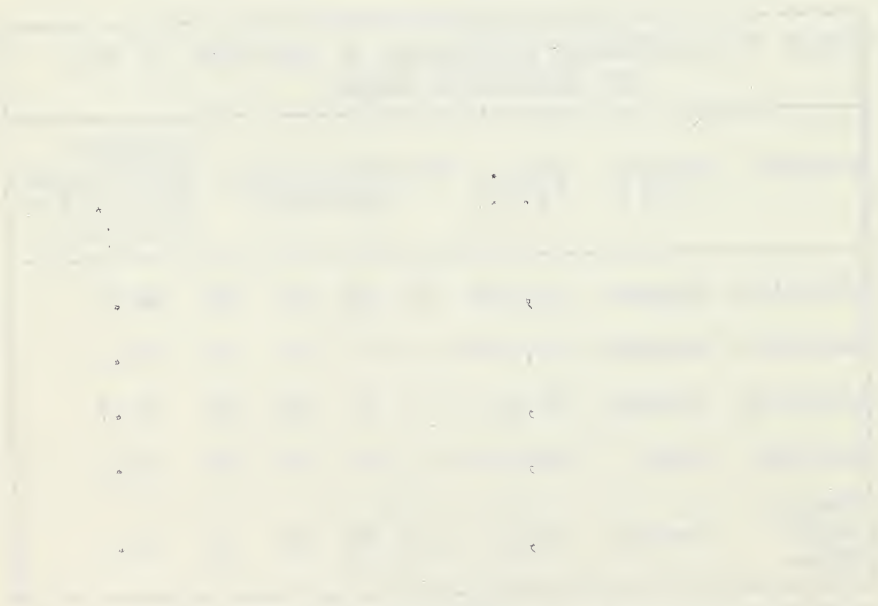
These conjugated estrogens are available in aqueous suspensions, oily capsules, suppositories and solutions in oil.

TABLE FORTY-SEVEN

ASSAY OF CONJUGATED ESTROGENS IN SOLUTION IN OIL BY TECHNIQUE THREE

Company	Vehicle (oil)	Conc. (I.U.)	N Galvanometer Readings				Average Determined Concs. % of the Known
Lakeside	Sesame	10,000	47	47	47	49	80.9
Lakeside	Sesame	10,000	45	46	47	46	75.4
Lakeside	Sesame	20,000	45	46	46	45	74.7
Squibbs	Corn	20,000	25	24	24	24	40.3
Sharpe and Dohme	Peanut	10,000	40	39	39	40	65.4

N Residue dissolved in sufficient alcohol to make a one milligram percent solution and 0.1 millilitre aliquots assayed by Technique Three.



The results of a series of extractions performed by the method of Carol and Rotondaro (71) and assayed by Technique Three are compiled in Table Forty-seven. In every instance the readings were far below the stated concentration of International Units of estrone.

The Photofluorometric method of assay does not appear to be applicable to the estimation of the potency of such estrogenic mixtures. The fact that the fluorescent phenomenon is not specific to any one estrogen and also that the estrogens vary in their biological potency would make difficult any statement regarding the true strength of these preparations based on a fluorometric assay.

SUMMARY

SUMMARY

1. A satisfactory photofluorometric method for the estimation of certain crystalline estrogens has been developed.
2. This method has been successfully adapted to the assay of commercial preparations of estradiol tablets, aqueous suspensions of crystalline estrone, and solutions of estrone in oil.
3. Efforts to develope a photofluorometric assay for urinary estrogens were unsuccessful.
4. The degree of fluorescence produced by testosterone, androsterone, dehydroandrosterone, dehydroandrosterone acetate and progesterone was considerably less than that produced by estrogens and has not been adapted to analytical procedures.

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